Control of feeding in Manduca sexta larvae

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Award date:
1988

Awarding institution:
University of Bath

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Submitted by William Andrew Timmins
for the degree of Ph.D. of the
University of Bath, 1988

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Abbreviations

AD	approximate digestibility
ANOVA	one way analysis of variance
BAPNA	N-α-benzoyl-DL-arginine-*p*-nitroanilide
C	digestible carbohydrate
Ci	Curie
cm	centimetre
CNS	central nervous system
CR	consumption rate
dpm	disintegrations per minute
ECI	efficiency of conversion of ingested food
ECD	efficiency of conversion of digested food
E+10	end of meal (includes bout criterion)
g	gram (except where indicated)
GR	growth rate
h	hour
kg	kilogram
L	lipid
LD	light: dark
m	metre
M	molar
MFO	mixed function oxidase
mg	milligram
min	minutes
ml	millilitre
mM	millimolar
n	number of replicates
N	normal
nm	nanometre
nM	nanomolar

nmol	nanomole
OD	optical density
p	probability level
P	protein
ppm	parts per million
S	start of meal
SD	standard deviation
SE	standard error
sec	seconds
TCA	trichloroacetic acid
Tr	retention time
µCi	microCurie
µg	microgram
µl	microlitre
v/v	volume by volume
W	watt
w/v	weight by volume
x	times

Acknowledgements

The work presented in this thesis was carried out in the Animal Physiology and Ecology Group at the University of Bath. It was supervised by Stuart Reynolds to whom I would like to express my great thanks for his expert advice. Financial support was from a Bath University Research Fund Bursary.

I would like to thank all of my family for their endless support and encouragement. My thanks to my contemporaries at Bath, expecially Andrew Bourke and Margaret Jones, and to the staff at 6W. Also, thanks to Sue Neil who helped with my amino acid analysis.

Abstract

Behavioural and physiological studies were performed on feeding and growth in fifth instar larvae of the tobacco hornworm, *Manduca sexta.*

Feeding was organised into discrete meals with no evidence of rhythmicity. The proportion of time spent feeding increased as the insects grew in size. This was due to longer feeding bouts and shorter gaps.

Larvae compensated for altered food quality by adjusting the amount eaten. Changes in bout length and gap length were associated with the compensatory response.

Evidence was found for a nutrient feedback mechanism operating in the control of meal initiation and termination. Volumetric feedback from the fore- and midguts was not demonstrated to be involved in the physiological regulation of feeding.

Azadirachtin impaired growth when injected into the haemocoel. Treated insects incurred increased metabolic costs and decreased nitrogen absorption from the ingested food. These effects were accompanied by apical swelling in the columnar cells of the midgut epithelium and a decrease in trypsin-like enzyme activity.

Overall, the study highlights the importance of nutrient levels to the growth and feeding physiology of *Manduca sexta* larvae.

Chapter 1

Introduction

A large number of insects have adapted to eat plants. Strong *et al.* (1984) have estimated that there may be more than 1/3 million species of phytophagous insects. It is not surprising that this vast diversity has prompted the speculation that most plant species endure at least some degree of insect damage (Crawley, 1983). Such damage is often most noticeable when insects eat plants that are grown on a large scale for human consumption. The destructive potential of crop pests has thus acted as an incentive to find ways of limiting the abundance and occurrence of certain insect species. Most of the strategies used have been primarily insect-directed. For example, the use of chemical insecticides and growth regulators, pheromonal disruption of reproduction and even biological control with natural enemies take little account of the pest's relationship with it's plant food.

More recently this relationship has received more attention as a factor in the coevolution of insects and plants (Ehrlich and Raven, 1964) and in determining the productivity of natural ecosystems (*e.g.* Mattson and Addy, 1975). Recent attempts have begun to use genetic engineering techniques to manipulate the relationship for crop protection purposes (*e.g.* Vaeck *et al.*, 1987). However, an important component of insect-plant relationships which has so far been comparatively neglected is the control of appetite in phytophagous insects.

The mechanisms controlling the intake of food have been examined in a few insect species and the only detailed work has been performed on blowflies and locusts (reviewed by Barton Browne, 1975a; Dethier, 1976; Bernays and Simpson,1982; Simpson and Bernays, 1983; Bernays, 1985). These studies have attempted to address the basic questions of what factors control the size and frequency of meals eaten by insects.

In brief, it seems that the control of meal size in blowflies is largely

dependent on negative feedback mechanisms associated with the volume of food in the gut (Dethier and Gelperin, 1967). The length of time between meals has been associated with the rate of gut emptying and a reduction in the inhibitory feedback from the gut. In turn, the rate of gut emptying has been linked to changes in blood composition after the meal, such as osmotic pressure (Thomson and Holling, 1977).

In locusts, volumetric feedback from the gut has also been strongly implicated in the control of meal size (Bernays and Chapman, 1973; Simpson, 1983; Roessingh and Simpson, 1984). There is a suggestion that the amount of food eaten in a meal by locusts may be regulated by a negative feedback process associated with total body volume (Moorhouse *et al.*, 1976). Also, increased blood osmotic pressure has been shown to influence meal size (Bernays and Chapman, 1974a; Roessingh *et al.*, 1985). As in blowflies, the control of meal initiation has been connected with a decrease in the negative feedback from the gut following feeding (Simpson, 1983). Changes in blood composition after feeding have been observed and recently a clear role for a nutrient feedback in the control of meal initiation has been demonstrated (Abisgold and Simpson, 1987). The behavioural pattern of feeding has been particularly well studied in locusts. An endogenous rhythm was discovered which underlied the tendency of locusts to start feeding and to commence several other behaviours (Simpson, 1981).

In contrast to blowflies and locusts, the regulatory processes involved in the control of feeding in Lepidoptera are almost competely unknown. Previous attempts to study feeding in caterpillars have only extended to the semi-quantitative examination of the temporal structure of feeding behaviour (Ma, 1972; Reinecke *et al.*, 1980). The results of these studies indicated that caterpillars do not feed continuously. This scarcity of information concerning the detailed organisation of feeding in lepidopteran larvae contrasts markedly with other aspects of the feeding process in caterpillars. For example, the perception of feeding stimulants and deterrents has inspired considerable attention (Schoonhoven and Jermy, 1977; Dethier, 1982; Blaney *et al.*, 1986).

It is clear that despite the great diversity of phytophagous insects, most plants are quite abundant and largely intact (Hairston *et al.*, 1960). Also, it is a general feature of herbivorous insects that they are not usually very abundant

(Lawton and McNeill, 1979). A commonly advanced explanation of this state of affairs is that plants have evolved several types of defence which have made it more difficult for insects to attack them. It is these defences which limit the success of herbivory. The physical structure of plant surfaces can deter and reduce feeding (reviewed by Southwood, 1986) and variations in the nutritional quality of plants can directly affect the growth and reproductive capacity of phytophagous insects (Southwood, 1973; Scriber, 1984). Secondary plants metabolites, or allelochemicals, were first postulated to possess a defensive function against insects by Fraenkel (1959). Since then, there has been considerable interest shown in identifying and extracting plant compounds for their potential pesticidal properties (Balandrin *et al.*, 1985). So far, roughly 10,000 allelochemicals have been chemically defined and it is believed that plants could yield as many as several hundred thousands of such chemicals (Swain, 1977; Harborne, 1982).

Some of these chemicals have been tested for their toxic and feeding deterrent qualities against insects (reviewed by Schoonhoven, 1982; Jermy, 1983). Among the most active plant compounds are members of the terpenoid class of plant products (Mabry and Gill, 1979). Azadirachtin is a terpenoid that is present in the fruits and leaves of the Neem tree (*Azadirachta indica*). It has been found to exhibit potent antifeedant and growth disrupting effects against many phytophagous insects (Kubo and Nakanishi, 1979). Such is the potency of azadirachtin that it has attracted much interest from development agencies in both developing and developed countries. However, despite much investigation little is known about the physiological mode of action of azadirachtin on the growth of insects.

In light of the paucity of information concerning the control of feeding in lepidopteran larvae, it was the aim of this study to apply some of the current theories of insect feeding to larvae of the tobacco hornworm, *Manduca sexta.* In addition, the effects of azadirachtin on the growth of *Manduca sexta* larvae were examined.

Chapter 2

The feeding behaviour of caterpillars (*Manduca sexta*) on tobacco and on artificial diet[1]

Introduction

The temporal analysis of feeding behaviour in insects has received little critical attention, a fact which is surprising considering the large quantity of work which has been carried out on other aspects of feeding, such as the underlying mechanisms regulating ingestion (reviewed by Barton Browne, 1975; Dethier, 1976; Bernays & Simpson, 1982; Simpson & Bernays, 1983), or the choice of host by the insect in relation to food quality (e.g. Dethier, 1976; Scriber & Feeny, 1979; Scriber & Slansky, 1981). Moreover, the analytical techniques for describing the temporal sequence of feeding behaviour have been well established from studies in vertebrates (e.g. Fagen & Young, 1978).

Virtually the only detailed studies of the pattern of feeding in an insect have been made on *Locusta migratoria*. This work is that of Blaney *et al.* (1973) and more recently Simpson (1982). Feeding was shown to occur in discrete bouts, separated by periods of 'roosting' where the nymphs climb their cage walls. Simpson (1982) also demonstrated a daily feeding rhythm, with reduced feeding activity at night.

An important difference between the feeding behaviour of lepidopteran larvae and Acrididae is that most caterpillars remain continually at the feeding site, whereas locusts leave their food between bouts.

We have studied temporal patterns of feeding behaviour in fifth instar larvae of a lepidopteran, the tobacco hornworm, *Manduca sexta,* feeding on

[1] This chapter is a slightly modified version of Reynolds, Yeomans and Timmins (1986). Additional paragraphs in this version are marked with an asterisk.

tobacco and on artificial diet. Jones & Thurston (1970) showed that some 90% of the food eaten by a *Manduca* larva during its life is consumed during the fifth stadium. The economics of food and water use during this stadium have been described by Reynolds *et al.* (1985), who suggested that the rate of food acquisition by *Manduca* caterpillars is optimized in order to maximize the rate of nutrient absorption from food in the gut and thus the rate of growth. In this paper it is shown that because *Manduca* caterpillars do not spend all of their time actively feeding, they could theoretically consume more food than they do. Changes that occur in the pattern of feeding according to the nature of the food and the size of the insect may provide some clues as to the way in which food intake is regulated.

Materials and Methods

*Rearing conditions**

Insects were maintained from hatching under standard conditions (see appendix 1) on artificial diet or growing tobacco plants (*Nicotiana tabacum*, var. 'White Burley')

Behavioural Observations

The feeding behaviour of day 1 fifth stadium larvae was observed directly on tobacco and artificial diet for 10 h (LD 5:5h). Larvae were placed on diet in clear plastic beakers (Pioneer Plastics, no. 10) on a white background with a glass plate as lid. Larvae were placed in pairs on tobacco plants. Records were made using a latching switchboard and event recorder (Rustrak) beginning 30 min after the observer had entered the room. Observations in the 'dark' were made under a dim red light.

To measure the amount of tobacco eaten per unit time, a leaf (with its petiole in water) was weighed before and after a caterpillar had spent 10 min actively feeding on it (*i.e.* time within bouts). For artificial diet a similar procedure was adopted.

*Statistical analysis**

Spearman rank correlations, paired t-tests and one-way analysis of variance(ANOVA) were performed using the Minitab 5.1 statistical package. MLP (maximum likelihood program; Ross, 1980) was used to fit data to negative binomial, log series and Poisson distributions. BMDP (biomedical program; Dixon, 1983) was used to fit autocorrelations to the data.

Results

Growth

Caterpillars feeding on tobacco plants and on the artificial diet grow at similar rates (Fig.2.1). However, their behaviour differs considerably. It is obvious even to the casual observer that the larvae on plants spend a great deal of their time engaged in active feeding (Heinrich, 1971, described feeding on tobacco as 'almost continuous'), whereas those on diet appear to be resting motionless on their food for much of the time. It was this casual observation that prompted us to examine feeding behaviour in more detail.

Description of feeding behaviour

On tobacco, the caterpillars feed from the sides of the leaves. Each sequence of feeding movements involves a series of bites (typically four to nine) which are directed downwards in an arc toward the animal, thus removing a strip of leaf. The bite rate during periods of active feeding was measured from videos on day 3 of the stadium and was found to be 1.90 ± 0.04 bites sec^{-1} (mean $\pm$SD, n=100, data from five individuals). There are two types of biting behaviour on artificial diet:

(i) Maximal biting. This is where the observed bite rate is quickest, as measured by single frame video analysis. The maximum bite rate was 1.84 ± 0.07 bites sec^{-1} (mean $\pm$SD, n=240, data from twenty-four individuals). Each sequence of feeding movements involved a series of bites (typically four to nine) occuring in a downward arc towards the prolegs. This is repeated many times during a feeding bout with each sequence lasting between 3 and 5 sec, depending on the number of bites performed. This pattern is essentially

the same as that seen on tobacco.

(ii) Exploratory behaviour. This describes a biting sequence where the gaps between bites are long. Typically, the animal is 'nibbling' at various sites on the diet; this is interpreted as exploration to find a suitable feeding site.

As both biting behaviours occur within any one feeding bout, the effective bite rate was less than the maximal rate described above. The effective bite rate was calculated by counting the number of bites during the entire feeding periods. The value obtained was 1.50 ± 0.08 bites sec^{-1} (mean $\pm$SD, n=240, data from twenty-four individuals). The bite rate of fourth instar larvae feeding on diet was found to be the same as for fifth instar animals at 1.50 ± 0.04 bites s^{-1} (mean $\pm$SD, n=40, data from four individuals).

Temporal analysis of feeding behaviour

Feeding on both tobacco and artificial diet was discontinuous (see Fig.2.2). Periods of active feeding (which are here called 'bouts') were separated by periods of inactivity (gaps). Gaps were also found within feeding bouts. This presents a difficulty in defining what consitutes a feeding bout and what constitutes a feeding episode within a bout. The problem was resolved by analysing the frequency of occurrence of particular lengths of gap between feeding events. This is now a standard technique in behavioural analysis (Slater, 1974; Fagen & Young, 1978) which was first applied to insect feeding behaviour by Simpson (1982).

Briefly, if individual feeding bouts are initiated independently with a probability that does not change with time, then it would be expected that the observed distribution of gaps in feeding would follow a negative binomial distribution. A simple test for such a distribution is to plot a log survivor curve. If the data approximates a negative binomial distribution, then the log survivor curve should be a staight line. However, if the observed gap lengths are derived from two distinct populations, there will be a discontinuity in the slope of the log survivor curve. The time at which this discontinuity occurs reflects the gap length where the two populations can be distinguished. This value is taken as the 'bout criterion'.

Examples of the distribution of gap lengths from individual larvae observed over a 10 h period are plotted as log survivor curves in Fig.2.3(A) for tobacco-fed caterpillars and in Fig.2.3(B) for diet-fed insects. All of the insects observed showed clear discontinuities in their log survivor curves, positioned between 1.5 and 2.5 min. Such inflexions are usually taken to indicate that there are two types of gaps. Those shorter than the bout criterion are 'intrafeed gaps', and represent events such as a change in feeding posture. Gaps longer than the bout criterion, on the other hand, reflect genuine periods of non-feeding, and are termed 'interfeed gaps'. It should be remembered that the bout criterion will not separate the two types of gap perfectly, since the distributions of their lengths will over-lap somewhat.

The position of the bout criterion for each insect was determined by inspection. The value of the bout criterion for tobacco-fed larvae was 1.6 ± 0.7 min (mean $\pm$SD, n=5) and for diet-fed larvae was 1.9 ± 0.5 (mean $\pm$SD, n=6). These values are not significantly different (t-test, $p>0.1$). Thus, broadly speaking, on either tobacco or artificial diet, a bout is any episode of feeding which is separated from any other such episode by a gap of more than 2 min.

It will be seen from Fig.2.3 that the distinction between intra- and inter-feed gaps is much clearer for insects fed on artificial diet than it is on tobacco. This is reflected in the much steeper slopes of the log survivor curves for interfeed gaps when tobacco is the food.

The lengths of the feeding episodes themselves may be examined by the same analytical procedures. Log survivor plots for the duration of the feeding events are given in Fig.2.4 for individual insects feeding on either tobacco or artificial diet. These plots showed no clear discontinuities of the kind discussed above, implying that all feeding events are of the same kind.

Feeding behaviour during light and dark

The data shown in Figs.2.3 and 2.4 were obtained from long observation periods (10 h) which spanned both light and dark periods of the photoperiodic regime. Although *Manduca* caterpillars are known to feed 'continuously' in both light and dark (and thus to grow at constant rate; Truman, 1972) we were concerned that there might nevertheless exist subtle differences in feeding

behaviour between dark and light periods. We compared the lengths of both interfeed gaps and bout lengths between dark and light periods of equal length (5 h) on day 1 for each individual. There were no significant differences between these parameters in light and dark for any of the six insects observed on artificial diet (t-test, $p>0.1$). This was also true for five of the six insects watched on the tobacco.

One insect, no. 6, showed significantly longer ($p<0.002$) interfeed gaps in the dark than in the light period which immediately followed, although bout lengths were the same ($p>0.1$). For this reason we have plotted log survivor curves of gaps separately for light and dark periods in the case of this insect (Fig.2.3A). Apart from this one case there is little reason to suppose that feeding behaviour differs between light and dark in *Manduca* caterpillars, and we have assumed in this paper that feeding behaviour observed during the light period is representative of the whole day.

On the probability of starting and stopping feeding

By using a χ^2 test for goodness of fit betweeen the observed distribution and a negative binomial distribution fitted to the data (Fagen & Young, 1978), it is possible to analyse further the data on the lengths of interfeed gaps (i.e. those longer than the bout criterion) to reveal non-random processes operating to initiate a feeding bout. Similarly, the observed distribution of the lengths of feeding events can be compared to a fitted negative binomial in order to check for non-random processes affecting the termination of feeding.

We used a computer program ('MLP') to check the goodness of fit of the data illustrated in Figs.2.3 and 2.4 to calculated negative binomial distributions. χ^2 values are given for each individual in the Figures. (The program also allowed us to check the data against Poisson and log series distributions: in every case the best fit was to the negative binomial distribution).

The observed distributions of interfeed gaps were mostly very close to the negative binomial model. All of six insects feeding on tobacco, and five of the six insects feeding on artificial diet showed behaviour that did not differ

significantly from that predicted by the negative binomial distribution (Fig.2.3). This may indicate that in most cases, the probability that an insect will begin to feed remains constant with time after the last bout. This is in marked contrast to Simpson's (1982) finding that most locusts showed an increasing tendency to resume feeding. It must be admitted that the small number of interfeed gaps observed for each caterpillar precludes any great confidence in this inference. The number of gaps that can be observed is constrained by the fact that feeding parameters change as the insect grows (see below). We did not feel it was wise to pool feeding data from observations on successive days. Similarly, differences between individuals make it undesirable to pool data from different insects (see Fagen & Young, 1978). Nevertheless, the data, such as they are, do not suggest any marked tendency to resume feeding.

Distributions of bout lengths are again mostly well described by negative binomials (Fig.2.4). This is particularly true for insects feeding on tobacco (five of six do not differ significantly from the predicted distribution). For those feeding on artificial diet, the fit is not quite so good. Although only two insects showed significant differences from the fitted negative binomial (one highly significant at $p<0.001$), almost all the log survivor curves have a distinctly convex appearance, and χ^2 values are relatively high. A good fit to the negative binomial distribution would indicate a constant tendency to cease eating once feeding has begun. However, as before, the rather small numbers of feeding bouts that it was possible to observe means that any such conclusion must be somewhat tentative.

Another test for non-random processes operating in the initiation and termination of feeding is to look for relations between the lengths of feeding bouts and the gaps which precede and follow them. The results of such an analysis are shown in Table 2.1, which records Spearman rank correlation coefficients for individual insects feeding on tobacco or on artificial diet. This procedure reveals that there is a strong dependence of feeding bout length on the duration of the gap which precedes it. Five or six insects feeding on tobacco showed significant correlations, and three of six feeding on diet. A similar correlation was found in 5th stadium larvae of the butterfly, *Pieris brassicae* (Ma, 1972). The dependence of gap length on the duration of the preceding feeding bout is much less marked. Only two of six insects on

tobacco showed a significant correlation in this respect, and only one of six feeding on diet.

The influence of the preceding gap on feeding bout length is further cause for our reservations about the goodness of fit of the bout data to the random model.

*Short-term rhythmicity associated with feeding**

An endogenous rhythm of feeding has been demonstrated in locusts (Simpson 1981) and it therefore seeemed worthwhile to examine our records of feeding in *Manduca* for such a short-term rhythmicity. A useful method of analysing data for sequential patterns is the Box-Jenkins (1976) time series analysis. This method measures the correlation between observations at different time intervals (lags) and expresses these as autocorrelation coefficients. With the exception of insect 9 on artificial diet, the distribution of observed gaps did not differ from a negative binomial suggesting that generally gaps are not of a typical length. This is unlikely to be characteristic of insects whose feeding behaviour is superimposed on an underlying oscillation. As a consequence, only data from insect 9 was tested for rhythmicity.

No clear evidence of a rhythm can be seen in the autocorrelogram (Fig.2.5). The peaks tend to be irregular and in no case exceed the 95% confidence intervals on either side of the autocorrelation coefficients.

Ontogeny of feeding behaviour in the fifth stadium

We have previously found that the amount of food eaten on successive days of the feeding period of the fifth stadium increases day by day (Reynolds *et al.*, 1985). To achieve this, feeding behaviour presumably changes.

Analysis of the behaviour of representative insects on each of these days showed that the bout criterion was always located between 1.5 and 2.5 min (data not shown). To expedite further analysis, we did not subsequently analyse feeding data according to individually-determined bout criteria, but made the simplifying assumption that all insects had the same bout criteria of

2 min.

We examined feeding behaviour during 3 h observation periods on each of the first 4 days of the feeding period (days 0-4) using the analytical techniques described above.

The percentage of time spent feeding on successive days of the fifth stadium (ignoring intrafeed gaps) is shown in Fig.2.6(A) for both tobaccco-fed caterpillars and those fed on artificial diet. The time spent feeding increases significantly through days 0-3 of the stadium for both tobacco (ANOVA, $p<0.005$) and artificial diet ($p<0.01$). Regression of time spent feeding against the caterpillars' fresh weights (Fig.2.7) shows that these parameters are highly correlated (r=0.72 for artificial diet).

The lengths of feeding bouts increase during the instar (Fig2.6B). There is a highly significant increase in bout length for both tobacco (ANOVA, $p<0.001$) lengthening of feeding bouts between day 0 and day 1, but subsequent days show significant increases in bout length.

In addition to these changes in feeding bout length, there are also changes in the durations of interfeed gaps (Fig.2.6C). On tobacco, gap lengths decrease from days 0 to 2 ($p<0.01$), remaining constant from days 2 to 3 ($p<0.05$). On diet, there is a significant decrease in gap length from day 0 to 1 ($0.05>p>0.01$), but no difference between the other days ($p>0.05$).

Thus for insects eating tobacco the increase in time spent feeding between days 0 and 1 is due to bouts being closer together. Consequently the frequency with which bouts are initiated ('bout rate', Fig.2.6D) increases ($p<0.05$). The large increase in time spent feeding between days 1 and 2 is due to bouts being closer together and longer, with no change in bout rate. A further increase in bout length on day 3 causes an increase in time spent feeding but no reduction is seen in the length of interfeed gaps and bout rate falls significantly on this day ($p<0.01$). In the case of diet-fed larvae the increase in time spent feeding from day 0 to 1 in the fifth instar results from both a slight increase in bout length and a decrease in interfeed gap length, resulting in a slight increase in bout rate (Fig. 2.6D). Increases in the time spent feeding from days 1 to 3 are through increases in bout length only, with

no difference in bout rate.

The percentage of time spent feeding within bouts did not change significantly as the larvae grew, whether tobacco or artificial diet was the food. Data from all four days have been pooled to give mean values of 85.2±5.2% (mean ±SD, *n*=35) for tobacco-fed caterpillars, and 82.0 ± 9.4% (mean ± SD, n=35) for artificial diet-fed larvae.

Rate of food acquisition

For the time spent feeding to be a valid measure of food intake, then the amount of food eaten in a set period must be constant under all the conditions studied. This was investigated by measuring the amount of tobacco or artificial diet eaten in 10 min of active feeding (*i.e.* within bouts) for fifth instar larvae of varying ages.

There was no significant variation with age in the amount of food eaten per unit time when caterpillars eating the same food were compared. However, much more artificial diet was eaten than tobacco. The rates of food consumption pooled for animals of all sizes are shown in Table 2.2. Because both effective and maximal bite rates are independent of age, and because the amount eaten is a product of bite rate and bite size, it follows that size of the bite is constant, and also that the two types of biting behaviour seen on artificial diet must occur in similar proportions through the instar.

Since the bite rates within bouts of feeding on the two foods are very similar (see above), this means that a bite of tobacco acquires less food than does a bite of artificial diet.

An estimate of the total amounts of each food eaten by a caterpillar in day 1 can be made from the behavioural data by multiplying the rates of food acquisition during bouts by the total time spent feeding per day. This has been done for day 1 fifth instar larvae in Table 2.2. The results show that caterpllars eat a considerably greater fresh weight diet of tobacco than they do of artificial diet. Because the water content of tobacco leaves (85%) was greater than that of the artificial diet (77%), estimates of the amounts of dry food taken in are much less different.

However, the dry weight of leaves eaten is still some 15% greater than the dry weight of artificial diet. Since the caterpillars grow in weight by a similar amount (Fig.2.1), growth on tobacco must be less efficient in dry weight terms than growth on diet- a conclusion that is in satisfactory accord with studies that have investigated the same question by more direct, gravimetric means (compare Waldbauer, 1964, with Reynolds *et al.*, 1985).

When the amounts of each food eaten in a single feeding bout are estimated by dividing the total food eaten per day by the total number of bouts, the fresh weight of tobacco eaten per bout is much more than the fresh weight of artificial diet. However, the dry weights of each food eaten per bout are virtually identical (Table 2.2).

Discussion

The temporal pattern of feeding

The general observations of fifth instar *Manduca* larvae feeding on tobacco made in this study are in accord with those made by other workers. Larvae feed while hanging beneath the leaves, a behaviour which has been suggested to be thermoregulatory in that the larvae are shielded from direct sunlight (Casey, 1976). This behaviour presumably also serves to reduce predation by birds and/or mammals. Handling of the leaves by the larvae we observed was similar to that described by McFadden (1968) and Heinrich (1971), with larvae feeding preferentially on leaf edges.

Our finding that feeding behaviour is restricted to distinct bouts contrasts with Heinrich's (1971) observation that feeding is 'almost continuous' for *Manduca* feeding on tobacco. However, in this study we observed that some larvae feeding on tobacco spend over 80% of their time eating, which might explain how Heinrich gained this impression. Ma(1972) has previously reported that caterpillars of the large white butterfly (*Pieris brassicae*) feed in bouts, although he did not analyse this quantitatively.

The components of feeding behaviour on tobacco and on diet are

apparently the same. In both cases feeding occurs in bouts. The criterion distinguishing pauses within and between bouts is similar in each case. Within bouts, the biting patterns on tobacco are similar to the 'maximal biting behaviour' described for larvae feeding on artificial diet, and bite rates for these behaviours were the same. Fourth instar larvae were in this respect similar to fifth instar ones. This uniformity in bite rate might suggest the existence of an underlying central oscillator controlling biting movements. However, it should be noted that sensory feedback is important in controlling mouthpart movements of the locust *Schistocerca gregaria,* an insect which also shows a rather constant frequency of biting under normal eating conditions (Seath, 1977).

The constancy of biting patterns during the fifth stadium and of the rate of food acquisition during bouts of active feeding, mean that these two important components of feeding behaviour are independent of body size. Perhaps this is not surprising given that the external dimensions of the caterpillar's head and mouthparts do not change through the fifth instar, despite the fact that the insect's weight increases by order of magnitude (Williams, 1980).

It was hoped that examination of the frequency with which gaps and bouts of particular length occur might indicate how the insect regulates its food intake. In the event, although a few insects clearly behave in a way that is distinguishable from a random model for the initiation and termination of feeding, most insects do not. This may simply reflect the fact that insufficient feeding events were observed to allow subtle deviations to be detected. However, it is certain at least that there is a strong random component in the beginning and ending of feeding bouts.

We did find, however, that there is a strong dependence of bout length (*i.e.* meal size) on the duration of the preceding interfeed gap. This was observed in more than half of all the insects examined. This finding implies that some internal variable changes in value with time since the insect last fed. This variable is a motivational factor that has little influence on the resumption of feeding, but which persists during the next bout of feeding and is an important causal factor in determining when the bout ends. McFadden (1968) suggested that *Manduca* caterpillars feed less during the morning. However, no evidence for a daily rhythm or an oscillation underlying feeding was found

in this study.

Feeding on tobacco and on artificial diet, and the regulation of food intake

The striking difference between feeding on tobacco and on artificial diet is the much greater proportion of the insect's time that is spent eating when tobacco is the food. Caterpillars fed on tobacco spend 3-4 times as long in feeding as those given artificial diet. The increased proportion of time spent feeding results mainly from a change in the time taken to eat a 'meal' (*ie*. bout length). The number of meals taken (measured as bout rate) is about the same in each case. This alteration in the temporal pattern of feeding allows the caterpillars to grow at the same (maximal?) rate on the two foods.

Similarly, as the caterpillars grow, they spend a progressively greater proportion of their time eating (whether tobacco or artificial diet) mostly because they take increasingly larger meals which take longer to eat. The number of meals taken changes rather little.

These two observations may provide clues to the ways in which food intake is regulated. Since meal size (*i.e.* bout length) is affected by the nature of the food (tobacco or artificial diet), this implies that the termination of feeding bouts is a result of the insect's ability to monitor some variable connected with the food itself. One such variable would be the volume occupied by the food within the gut. Volumetric feedback from gut stretch receptors is important in regulating meal size in locusts and flies (Simpson & Bernays, 1983). Volumetric feedback cou ld account for the dependence of bout length on the previous gap length. Since the caterpillar's midgut contents are continually removed, either by absorption, or by being packed into the ileum by the activity of the pyloric musculature (Reinecke & Adams, 1977), the extent to which the midgut wall is stretched will decline with time during each interfeed gap. Thus a long interfeed gap means that more food will be required at the next meal to stretch the gut wall, and so the meal will be longer.

At first sight this volumetric feedback hypothesis does not seem to fit the data relating to meal size. The estimated fresh weight (volume) of tobacco eaten per meal is almost 60% larger than the estimated fresh weight of

artificial diet eaten per meal (Table 2.2). However, the estimated dry weights of tobacco and diet eaten per meal are virtually identical. This is because the water content of the leaves (85%) is greater than that of the artificial diet (77%). However, since the water content of food in the midgut is regulated quite closely in the face of marked differences in the water content of the food before it is eaten (K. Bellward & S.E. Reynolds unpublished observations), the dry weight of a meal is likely to be the best predictor of its volume within the midgut.

The progressive increase in meal size as the insects grow larger may also be a consequence of volumetric feedback regulation of bout termination, since the amount of food accommodated within the midgut for a given degree of stretch in the midgut wall will be greater as the gut's total volume increases. On the other hand, since the frequency with which meals are initiated (bout rate) is apparently little affected by the nature of the food, it might be supposed that the initiation of feeding depends largely on factors intrinsic to the insect. Actually, the coincidence of bout rates on tobacco and artificial diet in these experiments is probably fortuitous; altering the composition of the artificial diet results, amongst other things, in a change in bout rate (Timmins *et al.*, 1988; chapter 3), implying that sensory feedback of some kind is capable of influencing the initiation of feeding, as well as it s termination.

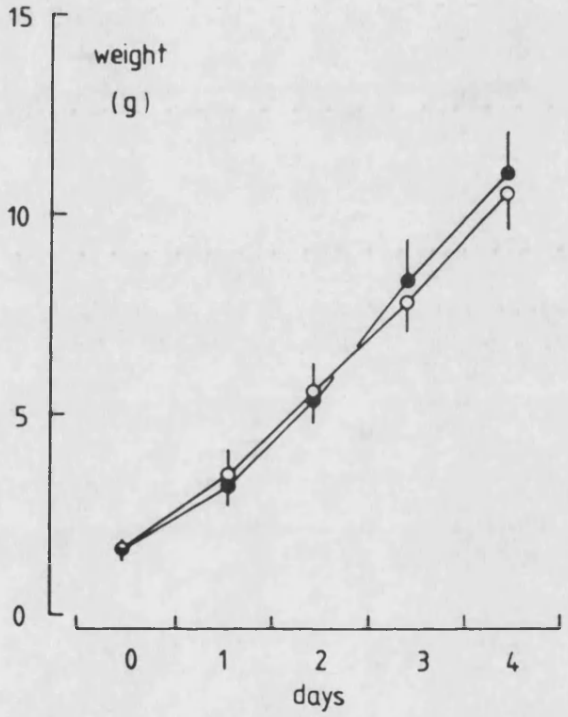


FIG.2.1. Growth in fresh weight of fifth instar *Manduca* caterpillars on tobacco (open circles; n=9) or artificial diet (closed circles; n=8). Means ± SE.

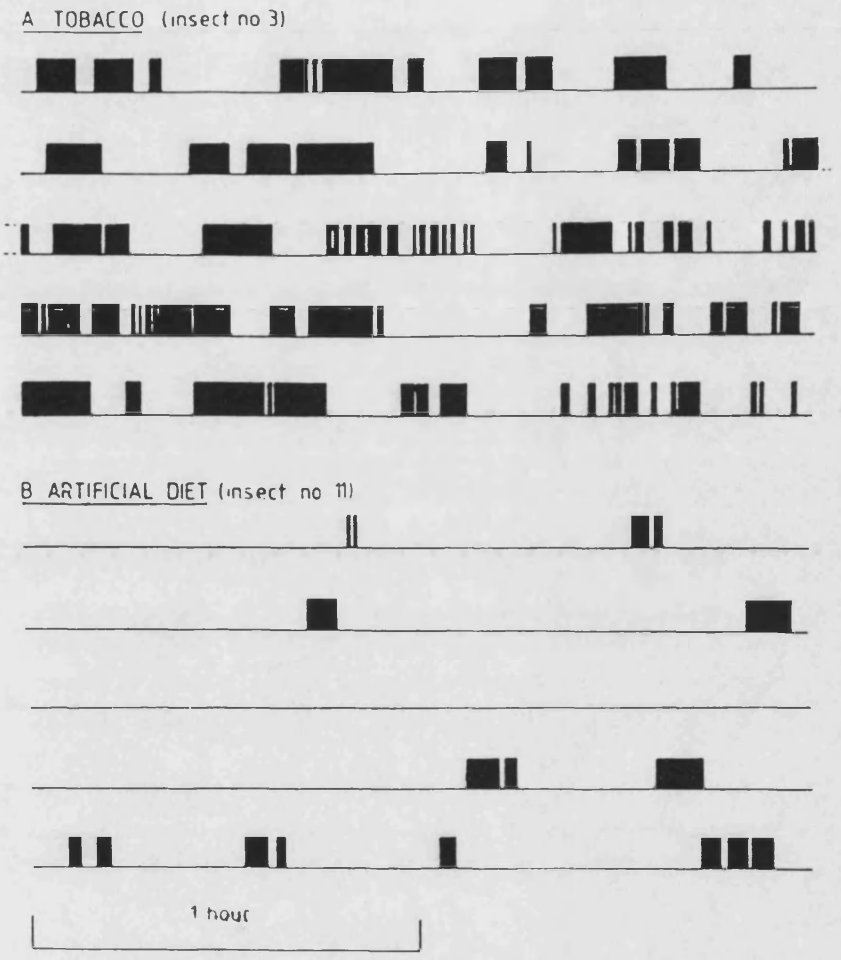


FIG.2.2. Sample records of feeding activity from (A) a single day 1 fifth instar caterpillar feeding on tobacco, and (B) a single caterpillar feeding on artificial diet. Periods of active feeding are shown as solid blocks. The records read from left to right. The records cover an observation period of 10 h in each case.

FIG.2.3. Log survivor functions of gap lengths for individual caterpillars feeding on (A) tobacco and (B) artificial diet. Each point shows the number of intervals that are longer than the gap length shown on the abscissa. The bout criterion (determined by inspection) for each insect is marked by an arrowhead. Each panel also gives the χ^2 statistic (and probability) describing the goodness of fit of the data for gap lengths longer than the bout criterion to a fitted negative binomial distribution (see text for details of how this was done). Individuals which differ significantly from the expected distribution are underlined. Data for insect 6 (tobacco) are shown separately for dark and light periods of observation, since these differed significantly in mean gap lengths.

NUMBER OF GAPS
A. TOBACCO
B. ARTIFICIAL DIET
MIN

FIG.2.4. Log survivor functions of bout lengths for individual caterpillars feeding on (A) tobacco and (B) artificial diet. Each point shows the number of bouts that were longer than the bout length shown on the abscissa. Each panel also gives the χ^2 statistic (and probability) describing the goodness of fit of the data to a fitted negative binomial distribution (see text). Individuals which differ significantly from the expected distribution are underlined.

A. TOBACCO
B. ARTIFICIAL DIET
NUMBER OF BOUTS
MIN
χ² (4df) = 2·79 P > 0·05
χ² (4df) = 5·93 P > 0·05
χ² (5df) = 0·98 P > 0·05
χ² (4df) = 3·18 P > 0·05
χ² (6df) = 15·93 P < 0·05
χ² (5df) = 3·38 P > 0·05
χ² (4df) = 14·91 P < 0·01
χ² (6df) = 8·80 P > 0·05
χ² (6df) = 2462·6 P < 0·001
χ² (10df) = 17·82 P > 0·05
χ² (12df) = 7·86 P > 0·05
χ² (10df) = 7·11 P > 0·05

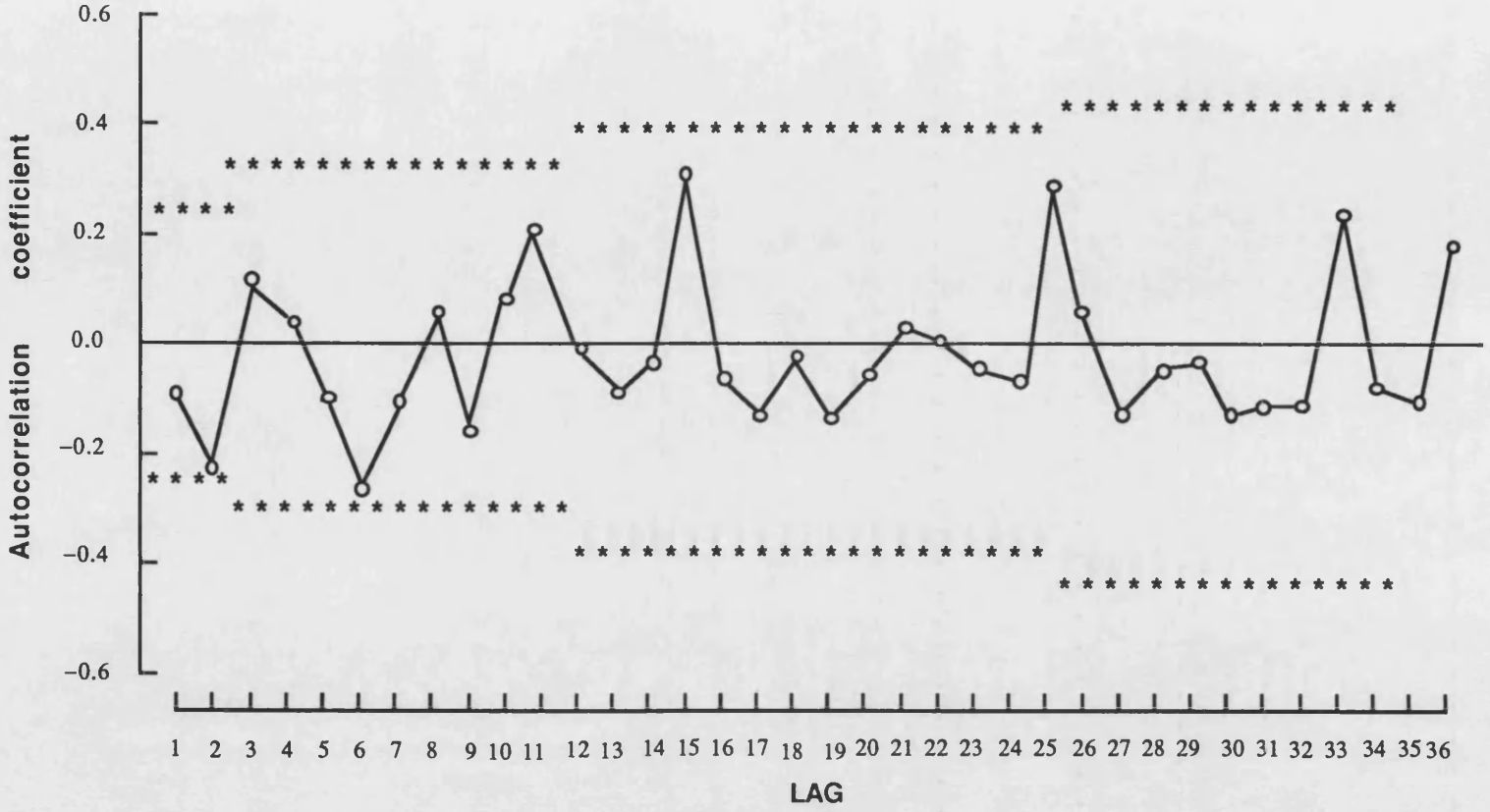


FIG.2.5. Autocorrelogram for insect 9 feeding on artificial diet.

*- Indicates 95% confidence intervals.

FIG.2.6. The ontogeny of feeding behaviour in fifth instar caterpillars feeding on tobacco, or on artificial diet. In each panel, the measured parameter is displayed in pairs of bars. Open bars represent data from tobacco-fed insects, solid bars artificial diet-fed insects. Each pair of bars represents one day during the feeding period of the fifth stadium. 'Day 0' is the day following ecdysis. (A) The time spent actively feeding (*i.e.* within bouts) as a percentage of the total time. (B) Bout length (min). (C) Length of interfeed gaps (min). (D) The rate of bout initiation (h-1). In each case the height of the bar represents the mean $\pm$ SE. Data was gathered from observations of nine larvae feeding on tobacco (six on day 0), and of eight larvae feeding on artificial diet.

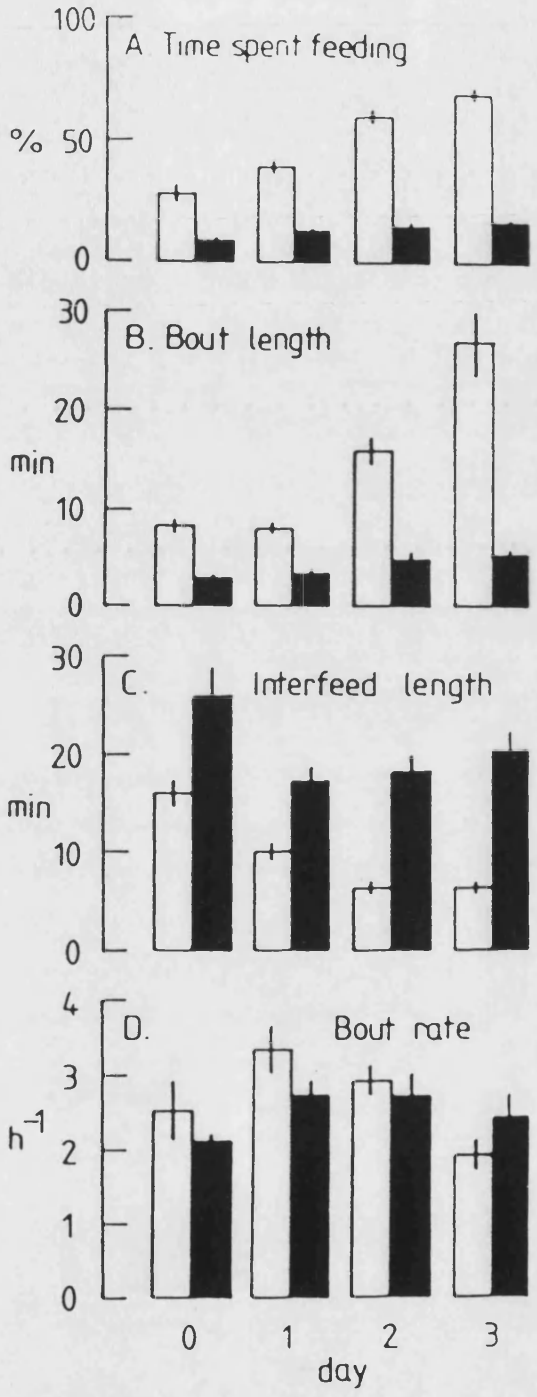

A. Time spent feeding
100
% 50
0
B. Bout length
30
20
min
10
0
C. Interfeed length
30
20
min
10
0
D. Bout rate
4
3
h⁻¹
2
1
0
0
1
2
3
day

FIG.2.6. The ontogeny of feeding behaviour in fifth instar caterpillars feeding on tobacco, or on artificial diet. In each panel, the measured parameter is displayed in pairs of bars. Open bars represent data from tobacco-fed insects, solid bars artificial diet-fed insects. Each pair of bars represents one day during the feeding period of the fifth stadium. 'Day 0' is the day following ecdysis. (A) The time spent actively feeding (*i.e.* within bouts) as a percentage of the total time. (B) Bout length (min). (C) Length of interfeed gaps (min). (D) The rate of bout initiation (h-1). In each case the height of the bar represents the mean $\pm$ SE. Data was gathered from observations of nine larvae feeding on tobacco (six on day 0), and of eight larvae feeding on artificial diet.

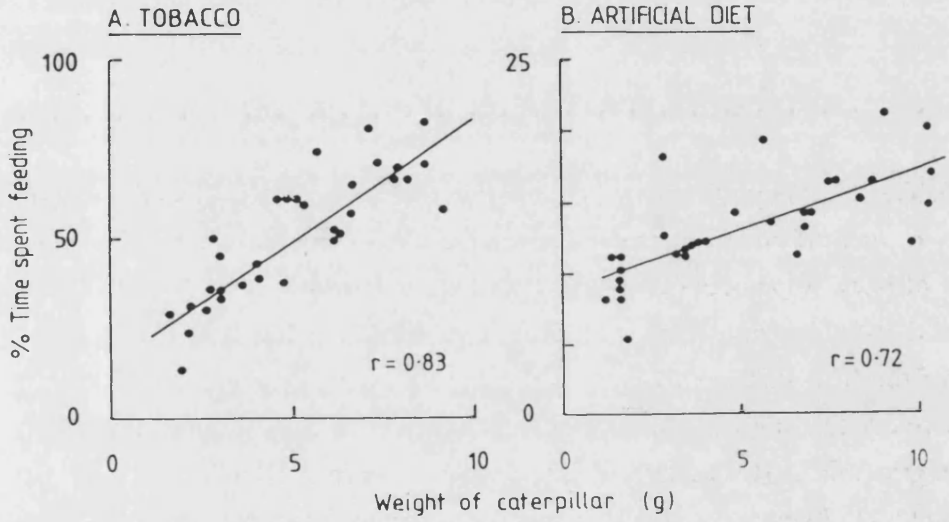


FIG.2.7. The percentage of time spent feeding on (A) tobacco and (B) artificial diet as a function of the caterpillar's fresh weight. The regression lines are drawn by the least squares method. The value of the correlation coefficient, r, is given in each case. Both are highly significant.

TABLE 2.1. Statistical analysis of the relation between the length of a feeding bout and the lengths of the interfeed gaps which preceded and followed it.

Insect no.	Spearman rank correlation coefficient (r_s) and probability (p)		n
	Bout length versus preceding gap	Bout length versus following gap	
Tobacco			
1	0.31 (p<0.005)	0.20 (p<0.05)	175
2	0.34 (p<0.001)	0.07 (NS)	199
3	0.26 (p<0.01)	0.11 (NS)	180
4	0.29 (p<0.005)	0.31 (p<0.005)	175
5	0.17 (NS)	0.16 (NS)	199
6	0.30 (p<0.005)	0.16 (NS)	175
Artificial diet			
7	0.44 (p<0.001)	0.04 (NS)	22
8	0.04 (NS)	-0.11 (NS)	16
9	0.01 (NS)	0.15 (NS)	32
10	0.27 (p<0.01)	0.17 (NS)	47
11	0.57 (p<0.001)	-0.05 (NS)	16
12	0.14 (NS)	0.44 (p<0.001)	18

TABLE 2.2. Amounts of food eaten by fifth instar *Manduca* caterpillars.

	Tobacco	Artificial diet
Measurement		
Fresh weight eaten per min[a]	0.011 ± 0.004 (15)	0.019 ± 0.002 (26)
Estimate		
Total fresh weight (g) eaten on day 1[b]	6.1	3.42
Total dry weight (g) eaten on day 1c	0.92	0.80
Fresh weight (g) eaten per meal[d]	0.082	0.052
Dry weight (g) eaten per meal[d]	0.012	0.012

[a]Measured. Mean ± SD (n). Pooled value for all sizes of caterpillar.

[b]Estimated from above,assuming times spent feeding on day 1 are 38.5% (tobacco) and 12.5% (artificial diet); see FIG.2.5A.

[c]Estimated from above,assuming dry weight contents of 15% (tobacco) and 23.5% (artificial diet).

[d]Estimated from above,assuming bout rates given in FIG.2.5D.

Chapter 3

Food intake, conversion efficiency, and feeding behaviour of *Manduca sexta* larvae given artificial diet of varying nutrient and water content[2]

Introduction

Faced with food that is quantitatively a poor source of nutrient, an insect herbivore has the options of moving to another plant or attempting to compensate for the reduced quality of its food. One way of compensating for poor food is to eat more of it. A number of insects compensate in this way (Scriber & Slansky, 1981). Such compensation is ecologically relevant because the quality of plants as food can vary seasonally (Scriber & Slansky, 1981), between species and cultivars (Slansky & Feeny, 1977) and between individuals (Scriber, 1977).

In this study we examined the behavioural and physiological responses of tobacco hornworm caterpillars to artificial diet in which nutrient content was altered by dilution with either cellulose (a presumed non-nutrient) or water.

Materials and methods

Rearing conditions and diet compositions

Insects were reared under controlled conditions (see appendix 1) until ecdysis to the fifth stadium when they were transferred to the experimental diets. The composition of the 'normal' diet was modified in these experiments by reducing the amounts of dry ingredients to 50%, 25% or 10% of the normal

[2]This chapter is a slightly modified version of Timmins, Bellward, Stamp and Reynolds (1988). Additional paragraphs are marked with an asterisk.

quantity and substituting cellulose powder (Sigma: C8002) while keeping water content the same, or by simply altering the water content of the mix to give diets with 200%, 50% and 25% of the normal concentration (Table 3.1). It should be noted that the final contents of water and dry matter in the diet did not change in simple proportion to the nutrient content of the diet, because not all of the dry ingredients were nutrients. Similarly, the proportion of dry weight that was attributable to nutrients varied between the diets that were diluted with water because the amounts of non-nutrient dry ingredients (agar and preservatives) were kept constant so as to preserve as far as possible the non-nutrient characteristics of the diet (*e.g.* texture).

Nutritional indices

Growth and food consumption were measured as described by Reynolds *et al.*(1985) until the day before wandering in the fifth stadium. The dry weights of animals at the beginning of the experiment were estimated by sacrificing another group of 10 caterpillars at day 0. Dry weights at the end of the experiment were determined directly by sacrificing the animals just before wandering.

The indices of food conversion efficiency given here are defined as follows:

Approximate digestibility (AD) (%) = 100 (E-F)/E

Efficiency of conversion of ingested food (ECI) (%) = 100P/E

Efficiency of conversion of digested food (ECD) (%) = 100P/(E-F)

where

E = dry weight of food eaten
F = dry weight of faeces produced
P = dry weight gain of insect

see Waldbauer (1968).

*Filming of feeding behaviour**

Feeding behaviour was videotaped while the caterpillars fed on diet in 50

ml pyrex beakers against a white background with a glass plate as lid. Larvae were filmed from directly above using a Hitachi CCTV (HV-17TK) video camera fitted with a Fujinon-TV zoom lens. All of the observations were made during the light part of the photoperiod. Additional lighting was supplied by 2 Thorn miniature fluorescent tubes each of 8W positioned approximately 50 cm above the beakers.

Observation periods lasted 13 h, of which the first 1 h was discarded to eliminate disturbances caused by the entry of the observer to the observation room. In each session the behaviour of 10 insects was recorded, beginning 24 h after transfer to the diet.

*Computer analysis of behavioural data**

Subsequently the videotapes were analysed by an observer who logged events directly with the aid of a microcomputer. A data collection program (see appendix 2) was written to use a BBC microcomputer as an event recorder. The times at which individual insects started or stopped feeding were stored on magnetic disk. Data files were transferred via a file transfer program (KERMIT, Lancaster University) onto a GEC 63/40 mainframe computer equipped with a UNIX operating system. The files were then analysed by a suite of sorting programs (appendix 2) to give the lengths of feeding events and non-feeding periods and to plot log-survivor functions for individual insects. Bout criteria were determined for each insect by inspection.

Briefly, the bout criterion is the minimum period of time which must elapse between feeding episodes before a period of non-feeding is considered to represent an interval between bouts. Shorter intervals are considered to be pauses within bouts (Reynolds *et al.*, 1986) and the length of a bout is computed as the total time actually spent feeding, and does not include intra-bout pauses. Similarly, the figure for percentage time spent feeding does not include intra-bout pauses.

Statistical analysis

Analysis (using the statistical package MINITAB 5.1) was by one-way

analysis of variance on log transformed data. Where this transformation was insufficient to produce normal distributions, the results were compared using a Kruskal-Wallace one-way analysis by ranks.

Results

Feeding and growth on food of varying nutrient and water content

When artificial diet was diluted with cellulose, fifth stadium *Manduca* caterpillars ate more of it (Table 3.2a). When the food contained only 50% of the normal amount of nutrient, the remaining 50% being made up with the cellulose, the insects ate about 1.5 times as much in both fresh and dry weight terms. Insects fed diet in which the nutrients had been diluted to only 25% of the normal level, further increased their food consumption (both fresh and dry matter) to 2.25 times the normal amount. These significantly increased consumption rates were insufficient, however, to compensate for the lowered levels of nutrients in these diets. Growth was significantly impaired, with insects on the 25% nutrient diet growing at only 43% of the control rate (Fig.3.1 and Table 3.2a). Caterpillars given diet diluted to only 10% of the normal nutrient content compensated less well and their rate of nutrient intake fell drastically. As a result their growth was very poor (Fig.3.2) and the experiment was terminated after 10 days.

Dilution of the diet with water gave a similar result in that the insects attempted to compensate for the reduced nutrient concentration of the food by eating a greater fresh weight of food (Table 3.2b). In this case, the extra consumption of the 50% nutrient diet (1.3 times in fresh weight terms) was not statistically significant, but the fresh weight of the 25% nutrient diet eaten (1.5 times normal) was significantly greater than usual. The extent of compensation on the 10% diet was less than for the 25% diet, but was still significant. When the water content of the diet was reduced to give 200% of the normal nutrient concentration the insects ate significantly less food in fresh weight terms (0.6 times control). The effect of these changes in the amount of food eaten was that the dry weight consumed did not differ from normal in the case of the 200% and 50% nutrient diets, but was significantly reduced in the

case of insects eating the 25% and 10% nutrient diets. When allowance was made for the differing proportions of dry ingredients in these di that were nutrients, only the insects given the 25% and 10% nutrient diets acquired significantly less nutrient than the control group. Only caterpillars fed the 50% nutrient diet were able to grow at the normal rate, however, implying that conversion of nutrients in the 200% diet was less efficient than usual.

The efficiency of food use

ECI is a simple measure of the overall efficiency of food use which relates the amount of growth to the amount of food eaten in dry weight terms. When the caterpillars' food was diluted with cellulose, the value of ECI (dry matter) was progressively reduced (Table 3.3a). As with ECI, the value of approximate digestibility, AD (a measure of the efficiency of uptake of food from the gut) fell as the amount of cellulose in the diet was increased. The extent of the reduction in AD was not as great as might have been expected for the 25% and 10% nutrient content diets (see Discussion).

ECD (efficiency of conversion of digested food) is a measure of the efficiency with which absorbed nutrients are used for growth. Since dry matter removed from food in the gut must by definition be nutrient, the value of ECD calculated from dry weights will automatically refer to the efficiency of nutrient utilisation. The values of ECD for normal diet and 50% nutrient diet diluted with cellulose did not differ significantly, but the ECD for insects eating 25% nutrient diet was significantly lower than normal. The extremely low value for ECD in insects given 10% nutrient diet indicates that almost none of the nutrients absorbed were used for growth.

When the water content of the diet was altered, ECI remained near normal for the 50% nutrient diet, but was significantl y decreased for 200%, 25% and 10% nutrient diets. Although AD showed significant variation between these diets, the extent of this variation was small compared to the changes seen in AD with cellulose-diluted diets.

ECD varied significantly between diets with altered water content, being lower than the value for the 'normal' artificial diet in every case. The extent of this reduction in ECD was less marked for water-diluted diets than for the cellulose-diluted diets. It is worth noting that decreasing the water content of

the diet was at least as deleterious as increasing it.

Retention times

An estimate of the retention time (T_r) of food in the gut may be made by dividing the dry weight of food present in the gut by the dry weight of food eaten per unit time (Reynolds *et al.*, 1985). Doing this calculation in terms of dry weight allows for the fact that the water content of food in the gut is different from that of the food before it is eaten, and also for the slight differences in the water content of the gut for caterpillars eating foods of differing water content.

Table 3.4 shows that when artificial diet is diluted with cellulose, T_r is significantly and progressively reduced. The increased speed of passage of the food through the gut is roughly proportional to the increased cellulose content. By constrast, when the dilutent is water, T_r is only significantly altered from normal when the extent of dilution is extreme (10% nutrient content).

Changes in feeding behaviour

The increased food consumption of caterpillars given diluted food results from an increased proportion of their time spent feeding. In a separate experiment from that described above, we observed the feeding behaviour of caterpillars that had been eating the experimental diets for 24 h prior to the 12 h observation period. Examples of some typical records are shown in Fig 3.3.

Table 3.5 gives some parameters of feeding behaviour on the different diets. The increased food consumption of insects eating diluted diets (see Table 3.2) is closely paralleled by increases in the time spent feeding. Similarly, when the nutrient concentration of the diet was increased by reducing the water content, the time spent feeding decreased.

Compensation involved changes in both the lengths of individual feeding bouts and the lengths of the gaps between feeding bouts. When the diet was diluted with cellulose the increased bout length and increased frequency of bout initiation contributed almost equally to the increased total of time spent feeding. For water-diluted diets, however, the main change in feeding

behaviour that contributed to the increased total time spent feeding was the greater length of individual feeding bouts. Although the lengths of interfeed gaps were significantly decreased the change was little more than the increased duration of the feeding bouts that preceded them. Thus the bout frequency was hardly affected.

Interestingly, the bout criteria of individual insects did not differ according to the nature of the food eaten, implying that the components of feeding behaviour remain unaltered despite changes in bout length. The difference between the bout criteria value determined here (about 3.5 min) and that found in a previous study (Reynolds *et al.*, 1986) is probably due to the different method employed for observing and recording feeding behaviour.

Discussion

Compensation of food intake for altered food quality

In this study, we have shown that fifth stadium caterpillars of the tobacco hornworm, *Manduca sexta* compensate for food which contains either more or less nutrients than usual. When their artificial diet is diluted either by replacing nutrients with cellulose, or by increasing the water content, the caterpillars attempt to maintain their intake of nutrient by eating more food. Conversely, when nutrient concentration is increased by decreasing the water content, the insects eat less food but maintain their intake of nutrients.

A number of insects compensate for poor food by increasing or decreasing consumption in this way; the Acridids *Schistocerca, Locusta* (Dadd, 1960; Simpson & Abisgold, 1985) and *Melanoplus* (McGinnis & Kasting, 1967) all show compensation for altered food quality, as do the cockroaches *Periplaneta* (Bignell, 1978) and *Blatella* (Gordon, 1968). The extent of compensation varies, but was particularly complete in *Melanoplus,* where nutrient intake was almost constant even when the diet was diluted sevenfold with cellulose.

Among Lepidoptera, experiments with both natural foods and artificial diets indicate that although at least some caterpillars compensate for poor food by increasing their intake,they do not always do so. *Pieris rapae* caterpillars

maintained their rate of nitrogen intake in the face of varying nitrogen content in different species of Cruciferous plants, and also in a single host species where nitrogen content was manipulated by the application of fertiliser (Slansky & Feeny, 1977). Similarly, Lawson *et al.*(1984) found that *Anisota senatoria* larvae feeding on mature oak leaves increased their intake of food to compensate for the differing nitrogen content of different oak species, so that their rate of growth was independent of leaf nitrogen. By contrast, *Alsophila pometaria* (which feeds on young leaves with a higher water content) did not compensate for variation of leaf nitrogen, so that growth rates on different oak species were directly related to leaf nitrogen. Tabashnik (1982) found that although *Colias philodice eriphyle* compensated for intraspecific variation in leaf nitrogen when eating vetch, it did not do so when eating alfalfa. The related *C. eurytheme* also failed to compensate for varying leaf nitrogen in alfalfa. Larvae of the arbivorous lepidopteran *Datana ministra* were found to show a higher ingestion rate on mature leaves of the American basewood compared with larvae offered leaves coated with a protein mixture (Schroeder, 1986). Celery leaves contain twice the amount of protein as the petioles and *Spodoptera exigua* larvae were shown to compensate for this difference by eating significantly more plant material when fed petioles as opposed to leaves (Griswold and Trimble, 1985).

In a study on artificial diet, *Celerio euphorbiae* caterpillars apparently compensated partially when given food diluted with water, although their intake of dry matter was not fully maintained (House, 1965). Reese and Beck (1978) on the other hand, found that black cutworms, *Agrotis ipsilon,* failed to compensate at all for dilution of their artificial diet with water, although they ate less of it when its water content was decreased. When *Bombyx mori* larvae were fed artificial diets with varying proportions of protein, they failed to compensate when the protein was soybean meal, egg albumen or a mixture of amino acids. Some compensation was seen when the protein was casein, and compensation was quite marked when gluten was used. This last diet, however, was very poorly utilised and the amount eaten was far less than for any of the other diets (Horie & Watanabe, 1983).

A general conclusion to be drawn from this brief review of experiments on Lepidoptera is that the occurrence and extent of compensation of food intake

for altered food quality varies between species of caterpillars, and also according to the nature of the food. In some cases compensation may be considerable, and is probably of ecological significance.

Feeding efficiency on food of altered nutrient content

It is scarcely surprising that dilution of the nutrients with cellulose (usually assumed to be a non-nutrient) led to marked decreases in the efficiency of absorption (AD) and overall efficiency of conversion (ECI). However, the fall in the value of AD was not as large as would have been expected. Indeed in the 10% nutrient diet our measurements indicate that almost 28% of the dry matter of the diet was absorbed as it passed through the gut, indicating that some non-nutrients had apparently been absorbed. It is possible that this apparent anomaly may be the result of inaccuracies in our estimation of the amounts of food eaten and/or faeces produced. As discussed by Schmidt and Reese (1986), even small errors of this kind can be multiplied during subsequent calculations. We suggest that it is improbable that *Manduca* utilises cellulose. A related Sphingid caterpillar has been found to be unable to digest cellulose (*e.g.* Santos *et al.*, 1983). By contrast, where the diet was diluted with water, AD hardly changed at all, despite a 2.6-fold variation in the fresh weight CR (Table 2). This doubtless reflects the fact that the rate of passage of food through the gut (measured as Tr) varies rather little for diets with altered water content (see Table 3.4), because the water content of the midgut contents is subject to regulation (K. Bellward and S. E. Reynolds-unpublished observations).

Low values of ECD reflect increased metabolic or other costs. When the artificial diet was diluted with cellulose, the values for the 25% and 10% nutrient diets were significantly reduced in relation to the normal and 50% nutrient diets. When the diet was diluted with water, ECD changed rather little for the 50% nutrient diet, but was markedly depressed for the 200%, 25% and 10% nutrient diets. Thus, diets with either lower or very much higher water contents than normal both led to increased costs and lower efficiency. Presumably at least some of these costs were associated with handling water within the body. When the food has a low water content, water must be resorbed from the faeces in order to maintain the insect in water balance. When the food has very much more water than usual, it may be necessary to

resorb salts. The high costs associated with increased cellulose content in the diet may be associated with increased activity during feeding. For both cellulose- and water-diluted diets the effect of increased metabolic expenditure on ECD will be compounded by the fact that because growth is slower the time taken to complete the feeding stage of the stadium is longer, and thus the total cost of basal metabolism will be greater.

A number of previous investigators have found an inverse relationship between the rate of eating (CR) and overall efficiency (ECI) (e.g. Slansky & Feeny, 1977, for *Pieris*; Tabashnik, 1982, for *Colias* ; Scriber & Slansky, 1981 for a number of species). All of these studies used intra- or interspecific variation in host plant leaf quality to effect the change in rate of intake. A problem here is that the interaction of rate and efficiency of food processing may depend on why the insect varies its rate of eating. More than one variable in food quality may alter in such studies; in particular, the proportion of dry matter that is nutrient (particularly nitrogen) is often positively correlated with water content (Scriber & Slansky, 1981).

Experiments with artificial diets have the advantage that the nature of the change in food consumption is known precisely. This study has shown that the addition of cellulose to the diet (which decreases the dry weight content of nutrients, but which does not change the balance between them) decreases ECI much more than does the addition of extra water. While the inverse relation between CR and ECI is borne out by the experiments with cellulose-diluted diets (at least, provided the dilution is not too great), this is not the case where the diet is diluted with water. As the water content of the food increases, dry weight) CR is at first maintained and then declines, while ECI is maximised for 'normal' diet and actually falls with the declining CR as water content is increased.

Changes in feeding behaviour

Feeding behaviour in *Manduca* caterpillars is organised into bouts of active feeding separated by periods of inactivity, whether the food is tobacco leaves or artificial diet. The principle difference between feeding behaviour on tobacco and on diet is that bouts are longer on tobacco. The frequency of bout initiation is similar for the two food (Reynolds *et al.*, 1986).

Among the many differences between tobacco leaves and artificial diet that might account for this difference in feeding behaviour is the much higher water content of tobacco compared with that of diet. We have suggested (Reynolds *et al.*, 1986) that bout length might be determined by the amount of dry matter eaten during the bout. This hypothesis was supported by the finding that the amount of dry matter acquired during a bout of feeding was the same for tobacco and diet-fed insects. The dependence of bout length on the dry weight (rather than fresh weight) of food eaten is consistent with a role for volumetric feedback from the gut in terminating feeding activity, and thus ending the bout. Such a system has been suggested to operate in *Locusta* (Simpson, 1983) and *Schistocerca* (Roessingh & Simpson, 1984). In *Manduca,* the midgut is the main reservoir of food in the body and a good candidate for the source of such volumetric feedback. We have shown (S.E. Reynolds & K. Bellward, unpublished observations) that the water content of the food in the midgut varies much less than the water content of the food itself, and thus the dry weight of food eaten is a better predictor of the volume that is occupies in the gut that it's fresh weight.

Nevertheless, factors other than volumetric feedback clearly influence feeding behaviour since food intake was increased when nutrients were diluted with cellulose. Increasing the cellulose content of the food alters the amount of nutrient to be obtained from a given bulk of food. We found that this led to significant changes both in bout length and bout frequency. Thus nutrient flow may have a role both in initiating feeding (thus influencing bout frequency) and in terminating it (thus influencing bout length).

In particular, the observation that bout length increases when nutrients (and hence phagostimulants) are in lower concentrations is interesting. This is true for both cellulose- and water-diluted diets. The prediction from what is known in other insects (Bernays, 1985) is that longer, larger meals (bouts) should occur when phagostimulants are in higher concentration. One hypothesis that might explain this is that in *Manduca* both volumetric and nutritional feedback are involved in the control of meal size. In this model, a decrease in the amount of nutrient ingested during a bout could be offset by an increase in the volume occupied in the gut by the food eaten during that bout. As it happens, the data from the cellulose-diluted diets fit this model very well,

with a simple summation of dry weight (*i.e.* volume) plus nutrient ingested per meal giving a constant value, perhaps corresponding to the notional set point of the system controlling meal size (Table 3.6). However, the data from the water-diluted diets do not fit this model at all, suggesting that either the fit for the cellulose-diluted diets is fortuitous or that additional factors come into play when the water content of the food varies. A more direct experimental approach will be needed to resolve this point.

Simpson and Abisgold (1985) recently studied the behavioural mechanism of compensation used by *Locusta*. The insects did not compensate when dietary carbohydrate was replaced by cellulose but they did increase their intake when protein content was altered in the same way. In this case more was eaten because the locusts took meals more frequently while meal size remained unchanged. The finding that dilution of a key nutrient with cellulose did not significantly affect bout length in locusts differs from our results with *Manduca,* perhaps indicating that the control of food intake may differ between these two insects.

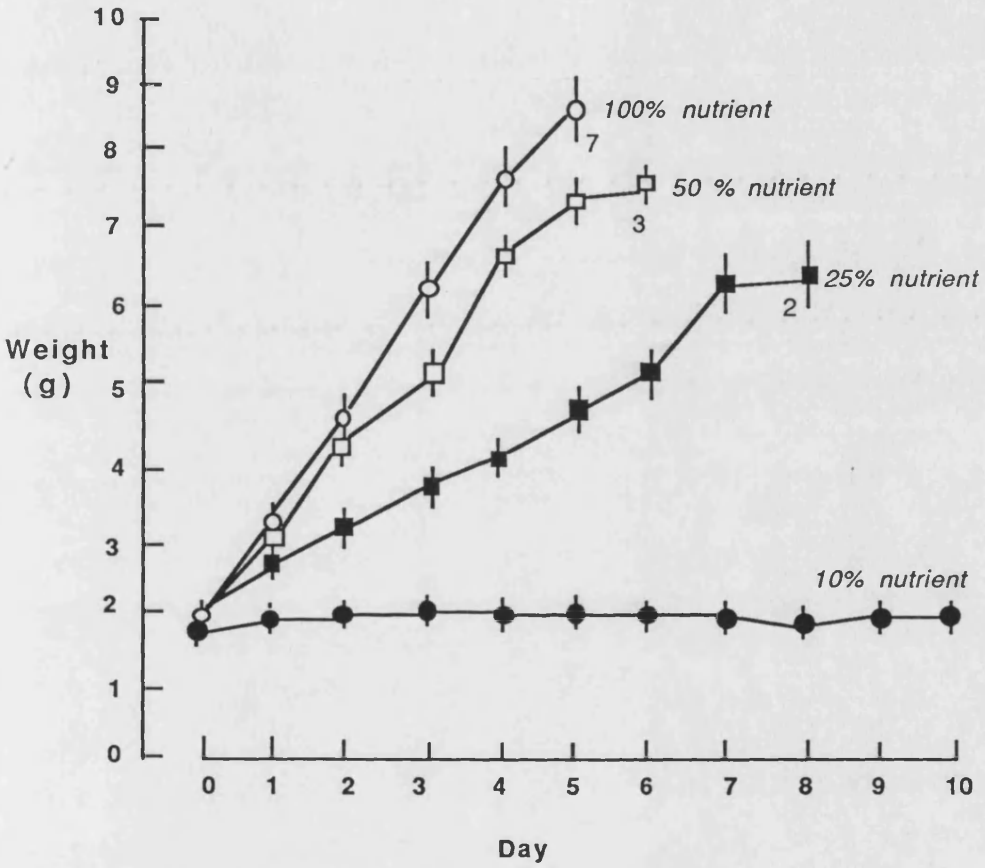


FIG.3.1. Growth of fifth stadium Manduca sexta caterpillars given diets diluted with cellulose. Means ± SE (n=10 per point except where indicated. Smaller sample numbers resulted when some insects ceased to feed and wandered).

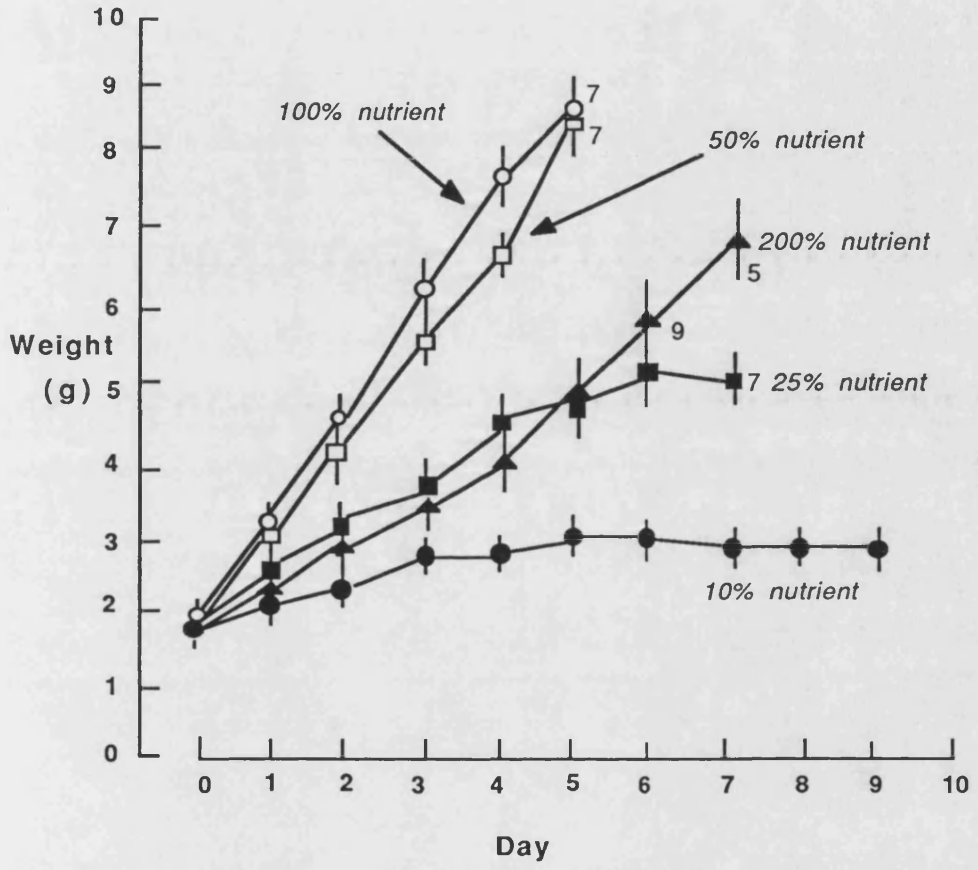
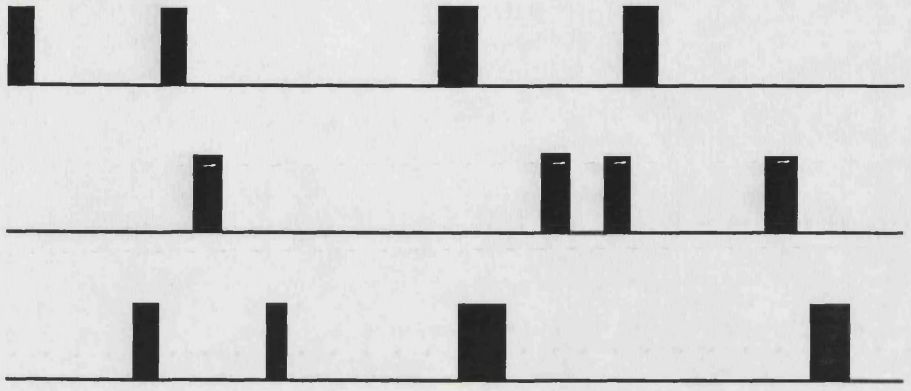


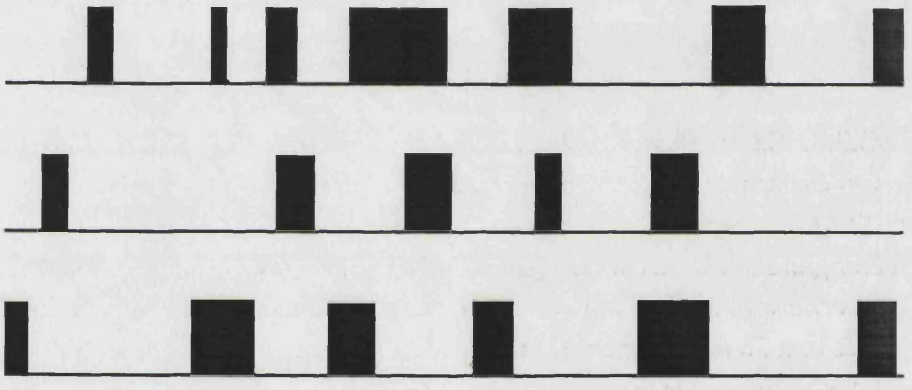
FIG.3.2. Growth of fifth stadium Manduca sexta caterpillars given diets diluted with water. Means ± SE (n=10 per point except where indicated. Smaller sample numbers resulted when some insects ceased to feed and wander).

FIG.3.3. Sample records of feeding activity from day 1 fifth stadium caterpillars given experimental diets as indicated. The insects were transferred from normal (100%) diet to the test diet 24 h before observations began. Periods of active feeding are shown as solid blocks. Gaps within bouts (*i.e.* those shorter than the bout criterion) are not shown. Each line represents the feeding activity of a single insect in a 2h sample period. (*N.B.* Data presented in Table 3.5 was from longer (12 h observation periods).

(a). Normal diet (100% nutrient)



(b). Diet diluted with cellulose (50% nutrient)



(c). Diet diluted with water (50% nutrient)

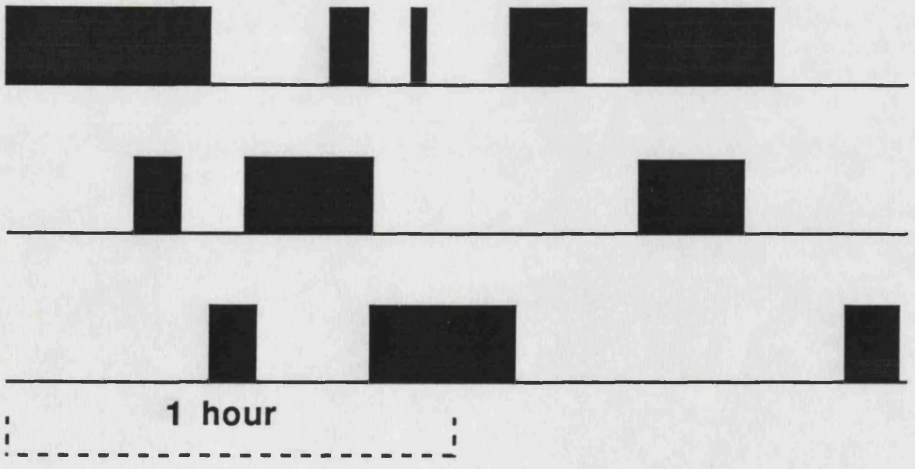


TABLE 3.1. Compositions of experimental artificial diets (means ± SE,n = 10).

(a) diets diluted with cellulose

	100% nutrient content (no added cellulose) (normal diet)	50% nutrient content (50% cellulose)	25% nutrient content (75% cellulose)	10% nutrient content (90% cellulose)
water (% of fresh weight)	80.44 ± 0.14	80.96 ± 0.03	80.24 ± 0.09	79.57 ± 0.17
dry matter (% of fresh weight)	19.56 ± 0.14	19.04 ± 0.03	19.72 ± 0.09	20.43 ± 0.17
nutrient (% of dry weight)	89.6	45.8	24.0	12.7

(b) diets diluted with water

	200% nutrient content	100% nutrient content (normal diet)	50% nutrient content	25% nutrient content	10% nutrient content
water (% of fresh weight)	66.81 ± 0.78	80.44 ± 0.14	85.43 ± 0.24	91.84 ± 0.02	94.69 ± 0.04
dry matter (% of fresh weight)	33.19 ± 0.78	19.56 ± 0.14	14.57 ± 0.24	8.16 ± 0.02	5.31 ± 0.04
nutrient (% of dry weight)	99.4	89.6	81.5	69.0	55.3

TABLE 3.2. Consumption rates (CR) and growth (GR) for fifth stadium *Manduca* caterpillars (day 0 to day before wandering) given artificial diets of varying nutrient and water content (means ± SE, n = 10).

(a) diets diluted with cellulose

	100% nutrient content (no added cellulose) (normal diet)	50% nutrient content (50% cellulose)	25% nutrient content (75% cellulose)	10% nutrient content (90% cellulose)
fresh CR (g/day)	2.86 ± 0.13^{a}	4.43 ± 0.14^{b}	6.40 ± 0.18^{c}	4.43 ± 0.14^{b}
dry CR (g/day)	0.56 ± 0.02^{a}	0.86 ± 0.03^{b}	1.26 ± 0.04^{c}	0.86 ± 0.03^{b}
nutrient CR (g/day)	0.50 ± 0.02^{a}	0.39 ± 0.01^{b}	0.29 ± 0.01^{c}	0.07 ± 0.003^{d}
fresh GR (g/day)	1.38 ± 0.06*	1.04 ± 0.05*	0.60 ± 0.03*	0.007 ± 0.01*
dry GR (g/day)	0.23 ± 0.01*	0.17 ± 0.01*	0.10 ± 0.01*	0.003 ± 0.001*

(b) diets diluted with water

	200% nutrient content	100% nutrient content (normal diet)	50% nutrient content	25% nutrient content	10% nutrient content
fresh CR (g/day)	1.59 ± 0.13^{a}	2.86 ± 0.13^{b}	3.65 ± 0.30^{bc}	4.17 ± 0.28^{c}	3.72 ± 0.13^{c}
dry CR (g/day)	0.52 ± 0.04^{a}	0.56 ± 0.02^{a}	0.53 ± 0.04^{a}	0.34 ± 0.02^{b}	0.20 ± 0.01^{c}
nutrient CR (g/day)	0.50 ± 0.04^{a}	0.50 ± 0.02^{a}	0.43 ± 0.04^{a}	0.24 ± 0.02^{b}	0.11 ± 0.004^{c}
fresh GR (g/day)	0.71 ± 0.05*	1.38 ± 0.06*	1.26 ± 0.05*	0.05 ± 0.04*	0.11 ± 0.01*
dry GR (g/day)	0.16 ± 0.01*	0.23 ± 0.01*	0.20 ± 0.01*	0.08 ± 0.01*	0.01 ± 0.001*

Significant differences within rows are indicated by different superscripts (one-way ANOVA, 95% confidence intervals based on pooled SD).

* Indicates that significant differences exist between means within the row (Kruskal-Wallis one-way ANOVA by ranks, $p<0.05$). This test was used where an F_{max} test on transformed data showed significant derivation from normality.

TABLE 3.3 The efficiency of food uptake and utilisation by fifth stadium *Manduca* caterpillars given artificial diets of varying nutrient and water content (means ± SE, n = 10).

(a) diets diluted with cellulose

	100% nutrient content(no added cellulose) (normal diet)	50% nutrient content (50% cellulose)	25% nutrient content (75% cellulose)	10% nutrient content (90% cellulose)
ECI	42.16 ± 1.33*	22.0 ± 1.10*	8.03 ± 0.30*	0.44 ± 0.20*
AD	50.55 ± 0.67*	26.91 ± 2.21*	20.44 ± 0.76*	27.56 ± 2.26*
ECD	83.59 ± 3.03*	78.12 ± 4.25*	40.02 ± 2.52*	1.97 ± 0.85*

(b) diets diluted with water

	200% nutrient content	100% nutrient content (normal diet)	50% nutrient content	25% nutrient content	10% nutrient content
ECI	31.78 ± 3.11*	42.16 ± 1.33*	40.35 ± 3.20*	24.11 ± 1.09*	2.28 ± 1.37*
AD	58.98 ± 4.45*	50.55 ± 0.67*	53.54 ± 2.46*	42.70 ± 2.24*	51.97 ± 1.94*
ECD	44.07 ± 6.54*	83.59 ± 3.03*	72.27 ± 5.99*	58.45 ± 4.93*	13.24 ± 1.24*

* Indicates that significant differences exist between means within the row (Kruskal-Wallis one-way ANOVA by ranks,$p<0.05$). This test was used where an F_{max} test on transformed data showed significant deviation from normality.

TABLE 3.4. Retention time (T_r) of food in the gut of day 1 fifth stadium *Manduca* caterpillars given artificial diets of varying nutrient and water content (means ± SE, n = 10).

(a) diets diluted with cellulose

	100% nutrient content (no added cellulose) (normal diet)	50% nutrient content (50% cellulose)	25% nutrient coontent (75% cellulose)	10% nutrient content 90% cellulose)
T_r (h)	7.13 ± 0.27^{a}	4.71 ± 0.35^{b}	3.57 ± 0.34^{c}	2.54 ± 0.07^{d}

(b) diets diluted with water

	200% nutrient content	100% nutrient content (normal diet)	50% nutrient content	25% nutrient content	10% nutrient content
T_r (h)	8.62 ± 1.03^{a}	7.13 ± 0.27^{ab}	7.33 ± 0.51^{ab}	6.08 ± 0.46^{b}	2.39 ± 0.16^{c}

Significant differences within rows are indicated by different superscripts (one-way ANOVA, 95% confindence intervals based on pooled SD).

T_r is estimated as dry weight of food in gut divided by dry weight of food eaten per h.

TABLE 3.5 Parameters of feeding behaviour in day 1 fifth stadium *Manduca* caterpillars given artificial diets of varying nutrient and water content (means ± SE, n = 10).

(a) diets diluted with cellulose

	100% nutrient content (no added cellulose) (normal diet)	50% nutrient content (50% cellulose)	25% nutrient content (75% cellulose)	10% nutrient content (90% cellulose)
Percentage of time spent feeding (%)	9.91 ± 0.38*	19.79 ± 1.40*	24.89 ± 0.81*	11.19 ± 1.01*
Bout length (min)	2.65 ± 0.11^{a}	4.67 ± 0.28^{b}	4.38 ± 0.12^{b}	3.46 ± 0.15^{c}
Gap length (min)	25.38 ± 1.37^{a}	19.41 ± 0.85^{b}	13.48 ± 0.57^{c}	26.25 ± 1.48^{a}
Bout frequency (h^{-1})	2.21 ± 0.11^{a}	2.42 ± 0.17^{a}	3.41 ± 0.12^{b}	1.92 ± 0.14^{a}
Bout criterion (min)	3.15 ± 0.36^{a}	3.85 ± 0.78^{a}	3.05 ± 0.39^{a}	2.25 ± 0.27^{a}

(b) diets diluted with water

	200% nutrient content	100% nutrient content (normal diet)	50% nutrient content	25% nutrient content	10% nutrient content
Percentage of time spent	5.53 ± 0.55*	9.91 ± 0.38*	13.20 ± 1.03*	20.94 ± 1.20*	9.51 ± 0.89*

feeding (%)

Bout length (min)	3.29 ± 0.18^{a}	2.65 ± 0.11^{b}	3.73 ± 0.13^{a}	5.18 ± 0.21^{c}	3.11 ± 0.13^{b}
Gap length (min)	56.53 ± 4.05^{a}	25.38 ± 1.37^{b}	22.63 ± 1.27^{b}	19.11 ± 1.04^{c}	26.75 ± 1.46^{b}
Bout frequency (h^{-1})	1.01 ± 0.11^{a}	2.21 ± 0.11^{bc}	2.09 ± 0.14^{bc}	2.43 ± 0.12^{b}	1.79 ± 0.17^{c}
Bout criterion (min)	4.10 ± 0.78^{a}	3.15 ± 0.36^{a}	4.20 ± 0.53^{a}	4.40 ± 0.39^{a}	3.80 ± 0.78^{a}

Significant differences within rows are indicated by different superscripts (one-way ANOVA, 95% confidence intervals based on pooled SD).

* Indicates that significant differences exist within the row (Kruskal-Wallis one-way ANOVA by ranks, $p<0.05$). This test was used where F_{max} test on transformed data showed significant deviation from normality.

TABLE 3.6 Estimates of dry weight and nutrients eaten during a single feeding bout for day 1 fifth stadium *Manduca* caterpillars given artificial diets of varying nutrient and water content.

(a) diets diluted with cellulose

	100% nutrient content (no added cellulose) (normal diet)	50% nutrient content (50% cellulose)	25% nutrient content 75% cellulose)	10% nutrient content 90% cellulose)
Dry weight eaten per bout (g)[1]	12	18	18	23
Nutrients ingested per bout (g)[2]	11	8	5	2
Sum	23	26	23	25

(b) diets diluted with water

	200% nutrient content	100% nutrient content (normal diet)	50% nutrient content	25% nutrient content	10% nutrient content
Dry weight eaten per bout (g)[1]	25	12	12	10	4
Nutrients ingested per bout (g)[2]	25	11	10	7	2
Sum	50	23	22	17	6

[1] Estimated from bout lengths (table 3.5), assuming a meal size of 12 mg for 100% nutrient content diet (Reynolds *et al*, 1986).

[2] Estimated from above, assuming nutrient content given in Table 3.1.

Chapter 4

Food intake, conversion efficiency, and feeding behaviour of *Manduca sexta* larvae given diets of varying carbohydrate, protein and lipid content

Introduction

Variations in food quality are known to adversely affect the fitness of insects (Scriber and Slansky, 1985). Many insects can respond to such changes by adjusting the amount of food they eat. In chapter 3, it has been shown that fifth instar larvae of the tobacco hornworm, *Manduca sexta*, compensated for alterations in the total nutrient content of their artificial diet by adjusting both the interval between feeding episodes and the length of feeding episodes.

The dietary requirements of insects include a complex mixture of different nutrients which are necessary for normal growth and development. The proper balance between these individual nutrients is often related to the particular life style of the insect (Dadd, 1985). The aim of this study was to examine separately the contribution of the carbohydrate, protein and lipid components of the diet to the growth of *Manduca* larvae and to investigate which are important in eliciting physiological and behavioural compensation.

Materials and Methods

Diet compositions

The carbohydrate and protein contents of normal diet were modified by

removing 50% or 100% of the normal quantity of sucrose or casein and substituting cellulose powder (Sigma:C8002) while keeping the water content the same (Tables 4.1a and b). Diets with reduced lipid content were prepared in a similar fashion by simply removing 50% or 100% of the normal quantity of cholesterol and oils (Table 4.1c).

Nutritional indices, behavioural and statistical analysis

The methods used were the same as those described previously in chapter 3.

Chemical analysis of diet and faecal samples

The digestible carbohydrate content of different diet samples and faeces produced by day 1 fifth instar insects was measured by the aniline test (Lorentz, 1963). 25mg of fresh diet or 10mg of dry powdered faeces was ground with a glass rod for 5min in an eppendorf tube with 0.5ml distilled water and then centrifuged at 3300 times g(force of gravity) for 5min. 0.25ml of the supernatant was withdrawn and the residue resuspended in 0.25ml distilled water. This procedure was repeated until 1.0ml of supernatant had been collected. 0.5ml of the supernatant was added to 5.0ml of aniline reagent (64.5mM aniline in glacial acetic acid) and boiled for 10min. The tubes were allowed to cool at room temperature for 0.5h and the optical density (OD) read at 405nm using a Cecil (CE 373) spectrophotometer. Sucrose standards were taken through the same procedure to produce a standard curve.

A Kjeldahl technique (Kjeltec system, Tecator) was used to measure the nitrogen contents of diet and faecal samples. 1.2g of dry powdered diet or 0.2-0.4g of dry powdered faeces were added to a tube containing 10.0 ml of concentrated H_2SO_4 and a catalyst tablet (3.5g K_2SO_4 + 0.4g $CuSO_4$). The tube was heated at 420°C for roughly 0.5h (until the mixture turned green) and then allowed to cool for 0.5h. 75ml of distilled water and 50ml of concentrated NaOH were added and the mixture steam distilled. 150ml of distillate was collected in a flask containing 25ml of a 4% boric acid solution and the distillate was titrated against 0.1N H_2SO_4 until the end-point was reached (pale yellow colour). A blank was run with each new batch of samples. The % content of each sample was calculated using the equation:

$$\% \text{ nitrogen content} = 14.01 \times \frac{(\text{ml titrant}_{\text{sample}} - \text{ml titrant}_{\text{blank}}) \times \text{normality of standard acid}}{\text{weight of sample (g)} \times 10}$$

The % protein content in dry weight terms was estimated from the % nitrogen content using a conversion factor of 6.6 (obtained by averaging the % nitrogen contents of casein and wheat germ protein). The limits of nitrogen detection and accuracy were investigated using $(NH_4)_2SO_4$ standards and was found to be 99% accurate in the range 0.5-50mg nitrogen.

Faecal nitrogen values were corrected for the presence of uric acid using the method of Buckner *et al.* (1980). 100mg of dry powdered faeces was mixed with 3ml of 200mm borate buffer (pH 8.5) and incubated in a shaking water bath at 60 °C for 0.5h. The mixture was centrifugedfor 10 min at 1600 times g(force of gravity) and the supernatant withdrawn. The residue was resuspended in 2.0ml buffer and the procedure repeated. The combined supernatants were made up to 50ml with buffer, 50µl buffer and 775µl distilled water were placed in a 1.0ml quartz cuvette and 100µl of uricase enzyme (Sigma:no.292-8) was added. The change in OD was followed on a Cecil (CE 272) ultraviolet spectrophotometer at 292nm until no further change could be detected (about 20min). Uric acid standards were taken through the same procedure to produce a standard curve. The uric acid content of the samples ranged from 0.5-0.7% dry weight.

A crude determination of the lipid content of samples was made by weighing following extraction by the method of Folch *et al.* (1957). 100mg of dry powdered diet or faeces was ground in a homogenising tube for 5min. with 2ml of a chloroform:methanol (2:1, v/v) mixture containing 0.05% butylated hydroxytoluene (acts as an antioxidant). The suspension was mixed (whirlimix, 30sec) with 0.4ml methanol and then centrifuged at 1600 times g(force of gravity) for 20min. The supernatant was decanted and placed in a tube which was weighed to 0.01mg. 0.8ml of chloroform and 0.6ml of a 0.88% KCL solution were added to the supernatant and mixed (whirlimix,

30sec). The solution was allowed to settle (5min) and the top layer removed and discarded. The remaining solution was dried down under reduced pressure (Speedvac,Savant) and the tube reweighed to give the weight of lipid in the sample.

For each diet, 5 determinations of carbohydrate, nitrogen and lipid content were made. Similarly, 5 determinations were also made for each type of faeces. Uric acid measurements were performed in triplicate.

Results

Feeding and growth on diets of varying carbohydrate, protein and lipid content

Fifth instar *Manduca* caterpillars did not eat more food when the carbohydrate content of the artificial diet was reduced (Table 4.2a, Fig. 4.1a). When insects were fed a diet containing 23% digestible carbohydrate (medium-C), they ate the same amount in dry weight terms as insects fed normal diet (27% digestible carbohydrate). The consumption rate (CR) was sufficient to maintain their intake of digestible carbohydrate, protein and lipid. Growth was not affected (Fig.4.2a). When the digestive carbohydrate content of the diet was further reduced to 19% (low-C) no increase in food consumption was seen despite the intake of digestible carbohydrate falling below the normal level. Insects consumed the same amount of protein and lipid on the low-C diet as insects feeding on normal diet and they grew at the same rate.

Similarly, reducing the protein content of the diet with cellulose did not elicit an increase in the dry matter CRs (Table 4.2b). Insects fed the 28% protein (medium-P) diet ate the same amount of protein, carbohydrate and lipid and grew at the same rate as insects given normal (30% protein) diet (Fig.4.2b). A small and non-significant increase in dry matter comsumption (1.1 times normal) was observed in caterpillars fed the 23% protein (low-P) diet. A clear increase in the amount eaten is detectable on day 0 of the 5th instar when 1.4 times more food ($p<0.05$, t-test) than normal was consumed by

insects fed the low-P diet (Fig.4.1b). No difference was found between the amount of protein, digestible carbohydrate and lipid eaten throughout the instar by insects given the low-P diet and those given normal diet. Even so, caterpillars fed the low-P diet grew at a significantly slower rate (Fig.4.2b).

Dilution of the lipid content of the diet with cellulose resulted in altered amounts of food being eaten (Table 4.2c). In the case of insects eating the 5% lipid (medium-L) diet, the consumption of food was significantly increased (1.2 times normal). This increase was particularly noticable during the second half (days 2-3) of the fifth stadium (Fig.4.1c). The amount of food eaten was such that intake of lipid and digestible carbohydrate was sustained at the normal level but the intake of protein was significantly increased (1.2 times normal). The increased protein intake was reflected in the higher growth rate of larvae fed this diet. Insects fed the 4% lipid (low-L) diet ate less food (0.7 times normal) and did not maintain a normal intake of digestible carbohydrate and lipid. The amount of protein eaten was similar to that eaten by insects fed normal diet but growth was still adversely affected.

The efficiency of food use

Table 4.3a shows the values of the approximate digestibility of dry matter (AD dry matter) and the approximate digestibility of individual nutrients for insects eating diets of modified digestible carbohydrate content. The value of AD(dry matter) refers to the overall efficiency at which nutrients are absorbed from food in the gut. It did not change as the carbohydrate content of the diet was reduced but AD(carbohydrate) was lowered, particularly on the medium-C diet. Nitrogen (*ie*. protein) was absorbed at the same efficiency on each diet but less lipid was removed from food of insects eating the medium-C diet. Values for ECI (efficiency of conversion of ingested food) and ECD (efficiency of conversion of digested food) are higher in caterpillars given the medium-C diet and lower in those fed the low-C diet (Table 4.4a).

A decrease in AD(dry matter) is seen on diets of reduced protein content (Table 4.3b). There was no significant variation in AD(carbohydrate) between the protein diets but AD(nitrogen) was decreased on the medium-P diet. The value of AD (lipid) was reduced to a greater degree than the other AD(nutrient) values and this was especially noticable on the low-P diet. ECI

was lowered on both of the diluted protein diets but ECD was lower only on the low-P diet (Table 4.4b).

When the lipid content of the diet was altered, AD(dry matter) remained normal. AD(carbohydrate) was significantly lower on the low- and medium-L diets. Nitrogen (*i.e.* protein) and lipid were absorbed less efficiently from the food in insects fed the medium-L diet (Table 4.4c). Insects given the low-L diet utilised the ingested food less efficiently for growth (reduced ECI and ECD values).

Retention times

Retention times (see Reynolds *et al.*, 1985) for each diet are shown in Table 4.5. Irrespective of the type of nutrients or the degree of dilution, food passes through the gut at a constant rate.

Changes in feeding behaviour

In a separate experiment to that described above, the feeding behaviour of day 5th instar caterpillars was recorded for 12h after transfer to normal, low-C, low-P and low-L diets.

The significant increase in the time spent feeding on the low-P diet (Table 4.6) is in agreement with the increased consumption (1.4 times normal) described previously on this diet (see Fig.4.2b). In fact, the behavioural data suggests that the compensation may actually be greater (1.9 times normal) than the value suggested by the gravimetric data.

This discrepancy could be explained by the insects eating more during the 12h of filming than later in the day and Simpson and Abisgold (1987) showed changes in interfeed length in locusts during 12h of observations. Alternatively, the time spent feeding may have been overestimated (*i.e.* the amount eaten per min might be lower). Nevertheless, the increase in food consumption on the low protein diet and lack of any compensation in insects fed the other low nutrient diets is confirmed.

The parameters of feeding behaviour obtained for insects fed the low-L diet were not significantly different from insects that had eaten normal diet despite the previously noted 27% decrease in CR on the low-L diet. This

could have resulted from either (or both) of 2 causes:(a) in this experiment consumption was not reduced; (b) (more likely) consumption on day 0 is reduced only slightly being progressively more severely affected later in the instar. The growth curve (Fig.4.2c) supports this latter hypothesis.

The nutritional composition of the food eaten did not affect the bout criterion in any case, although, the values presented here for day 0 fifth instar *Manduca* are greater than those determined previously using identical methods for day 1 5th instar *Manduca* (see chapter 3). This implies that the components of feeding behaviour may differ between the first 2 days of the instar with fewer short gaps occurring between meals in the feeding pattern of day 0 caterpillars.

The main component of the increased time spent feeding exhibited by insects fed the low-P diet was an increased bout frequency (1.7 times normal). But an increase in bout length (1.3 times normal) was also obseved. Bout length was significantly increased on the low-C diet (1.4 times normal) but this increase was insufficient to produce a significant increase in the overall time spent feeding.

Discussion

Effects of dietary nutrients on growth and food intake

When *Manduca* larvae were fed on diets of reduced protein content they maintained their intake of protein by a slight increase in food intake but this was insufficient to prevent a decrease in growth rate on the diet of least protein content (low-P). When insects were fed lipid deficient diets an increased food intake was noted on the medium-L diet but compensation failed to occur and growth rate was reduced on the low-L diet. Food consumption and growth were unaffected by dilution of the digestible carbohydrate content of the diet.

A substantial amount of evidence suggests the importance of dietary protein to the abundance, performance and control of feeding in insects (see reviews by Mattson, 1980; Scriber and Slansky, 1981; Scriber, 1984; White, 1984). The level of dietary protein seems to be important to the growth of *Manduca* caterpillars and compensation has been observed in several other

species of Lepidoptera (see chapter 3).

Evidence for compensatory changes in food intake in response to changes in dietary carbohydrates has been shown in several groups of insects, for example cockroaches (Bignell, 1978; Gordon, 1968), blowflies (Gelperin and Dethier, 1967) and fruit flies (Nestel, *et al.* 1985). The lack of compensation shown by *Manduca* larvae to a decrease in the level of dietary carbohydrate either indicates that the caterpillars cannot detect a decrease in the carbohydrate content of the diet or that the dilutions used in this study were insufficient to warrant compensation. The low-C diet contains 70% of the digestible carbohydrate content of normal diet and locusts are known to show a lack of compensation for a 50% decrease in dietary carbohydrate (Simpson and Abisgold, 1985). The nature of the *Manduca* diet means that it would have been very difficult to reduce it's content of digestible carbohydrate further without also affecting the content of other nutrient classes.

Lipids are necessary to insects as a source of energy, as components of cell membranes and as precursors to moulting hormones. A few studies have looked at the effects of dietary cholesterol on the survival of insects and the results are variable. For example, the survival of *Heliothis zea* was increased as the concentration of cholesterol in the diet was increased (Ritter and Nes, 1981), but a similar increase reduced survival in the adults of the dipteran *Hylema brassicae* (Dambre-Raes, 1976). This study has shown that *Manduca* larvae are sensitive to the lipid content of the diet and can compensate for a medium dilution by increasing their food intake. At lower dilutions, larvae do not attempt to compensate and are perhaps unable to meet the metabolic cost of increased food consumption.

In comparison with the degree of altered food intake seen when the overall nutrient content of the diet was altered without altering the proportions of individual nutrients (see chapter 3), the compensatory repsonses to dilutions of specific nutrients were considerably less. In this study, food intake was only significantly greater than normal throughout the instar in insects fed in the medium-L diet. There are several possible explanations for this result. Several nutrients were reduced together in the previous study and the dilutions used were also greater. In this study, the largest reduction in the level of a specific nutrient was associated with the low-L diet which contained only 66%

of the lipid content of normal diet. In comparison, a lower range of overall nutrient contents (50, 25, and 10%) were possible in the diets diluted with cellulose or water. It is possible that in the present experiments where no compensation was observed the reductions in the levels of carbohydrate, protein and lipid were not sufficient to 'nutritionally stress' the insect. It is clear though that at least in the cases of the low-P and low-L diets food quality was sufficiently reduced both to adversely affect growth and to stimulate compensation.

Vertebrates are also known to adjust their food intakes in response to variations in the levels of dietary nutrients. Rozin (1968) showed that rats can compensate for dilution of dietary protein and this response has also been demonstrated in hamsters (DiBattista, 1987). In addition, rats show the ability to select between diets of different protein contents and eat a mixture which sustains normal growth (Leathwood and Ashley, 1983; Sanders *et al.*, 1984). The dilution of sucrose solutions has also been shown to induce a compensatory increased intake in rats (Collier and Bolles, 1968) and a similar increase in consumption has been found in response to dilution of the lipid content food sources (Castonguay *et al.*, 1984).

Thus insects are similar to higher animals in their ability to detect and compensate for nutritional deficiencies. The fact that *Manduca* is sensitive to deficiencies of protein and lipid, but apparently less to a deficiency of digestible carbohydrate, may reflect the fact that these nutrient classes are much more likely to be limiting in its normal food (leaves of Solanaceous plants) than are carbohydrates.

Effects of dietary nutrients on feeding efficiencies

The overall efficiency of nutrient absorption (AD dry matter) was reduced in insects given the protein diluted diets but it remained normal in those fed the carbohydrate and lipid diluted diets. Values of AD for individual nutrients either remained the same as normal or were reduced but no increases were observed. ECI and ECD tended to decrease as the amount of individual nutrients in the diet was reduced.

Insects given food of poor quality also have the opportunity to compensate

by increasing the efficiency of absorption and utilisation of ingested nutrients. A slower rate of passage of food through the gut would be expected to lead to an increase in the amount of nutrients absorbed. In the event, retention times for day 1 larvae showed no tendency to increase in any of the experimental groups (Table 4.5). Likewise, no increase in ECI or ECD was observed although it is possible that *Manduca* larvae increased the conversion efficiency of individual nutrients into larval biomass. Unfortunately, it was not possible to estimate these parameters for individual nutrients. The results agree with those described in other species of Lepidoptera of a general decrease in AD, ECI and ECD with reduced food quality (reviewed by Slansky and Scriber, 1985).

Insects fed diets containing the least amount of each nutrient all showed reduced ECD values and therefore might have incurred some extra metabolic costs. A possible candidate for such costs includes the energy expended on maintaining a normal balance of nutrients in the insect's body. For example, an altered balance of nutrients in the diet might necessitate a compensatory increase in enzyme synthesis and thus energy expenditure. The altered balance of nutrients in the gut might lead to an unbalanced mixture of nutrients being absorbed. This might lead to increased costs. For example, insects fed the low-P diet might absorb a relative excess of carbohydrates and lipids leading to excessive concentrations of these nutrients in the haemolymph. These would then need to be catabolised. At the moment, it is only possible to speculate on the nature and importance of such factors and a more detailed investigation involving the measurement of respiration rates, enzyme levels and nutrient flows would be required to answer this question.

In recent years, information has started to accumulate on the self-selection of nutrients by immature insects. Waldbauer *et al.* (1984) showed that *Heliothis zea* larvae self-selected an 80:20 ratio of protein to carbohydrate when offered artificial diets lacking either sucrose or casein. Larval growth was greatest when insects were fed a diet containing the chosen 80:20 ratio. Cohen *et al.* (1987) found that the cockroach, *Supella longipalpa* preferred a carbohydrate-rich diet (20:80, casein:glucose) but that nymphs grew poorly when fed a mixed diet containing this ratio. In order to explain this result, it was suggested that the daily nutrient requirements of the nymphs changed through the instar with the need for carbohydrate being expecially high at the

beginning of the instar. Zucoloto (1987) demonstrated a preference for protein in young larvae of the dipteran, *Ceratitis capitata* , and a similar protein preference could explain the increased amount of low-P diet eaten by day 0 caterpillars (Fig.4.1b).

In a comparitive study, Simpson *et al.* (1988) confirmed the protein preference shown by *Heliothis* in another lepidopteran (*Spodoptera littoralis*) and also the carbohydrate prefrence shown by *Supella* in another orthopteran (*Locusta migratoria*). A difference in preference between these 2 insect groups could be explained by the requirement for protein by caterpillars in order to maintain their high growth rate and for carbohydrate by acridids and cockroaches to synthesise the structural carbohydrates present in the cuticle (Bernays, 1986). Additionally, as previously mentioned it is possible that these preferences reflect the composition of their natural foods.

The behavioural and physiological responses of an insect to changes in the levels of dietary nutrients is therefore likely to be influenced by varying nutritional demands both within an instar and also between different species of insects.

Changes in feeding behaviour

Caterpillars given diets of different carbohydrate, protein and lipid contents altered the structure of their feeding activities. Insects ate larger meals on low-C and low-P diets and took meals more frequently on the low-P diet. Nymphs of *L. migratoria* show a similar increase in meal frequency on diets of lowered protein content but meal size is not altered (Simpson and Abisgold, 1985).

The length of a feeding bout is influenced by the level of chemoreception from the food and insects respond to a number of different phagostimulants (Schoonhoven, 1981). Sugars are effective phagostimulants for many insects, including *Manduca* (Städler and Hanson, 1978), and amino acids stimulate feeding in another lepidopteran, *Pieris brassicae* (Ma, 1972). One might, therefore, expect a decrease in bout length as the level of phagostimulants in the diet is lowered by dilution with cellulose. The fact that an increase was observed in the present experiments is interesting and several possible

explanations can be forwarded.

It is possible that the carbohydrate and/or protein content of normal diet, at least at higher concentrations, exert an inhibitory influence on bout length so the bout length increases on low-nutrient diets. Ma (1972) found that the duration of the first 'feeding episode' of *P.brassicae* larvae was less on a diet containing 1.0M sucrose compared with one containing 0.1M sucrose. The reliability of this result is questionable since a bout criterion was not used to define the end of a meal but faecal production was also measured on the different sucrose diets and found to be lower on the 1.0M sucrose diet. Evidence to support this hypothesis is also available in acridids and the meal size of *Locusta* nymphs is reduced on pith discs impregnated with concentrations of sucrose greater than 0.125M (Cook, 1976). The sucrose concentration of normal diet can be roughly estimated from the amount of sucrose in the diet (appendix 1) and from the % digestible carbohydrate (dry weight) of the diet. Such a calculation produces a figure of 0.1M sucrose for normal diet. This is likely to be a considerable underestimate of the total digestible carbohydrate concentration because the wheatgerm in the diet also contains about 50% dry weight of carbohydrate (Geigy, 1956). Thus, the calculated carbohydrate concentration is similar to the sucrose concentration required for inhibition in *P. brassicae* and *L. migratoria.* Unfortunately the free amino acid contents of the diets used in these experiments were not determined but it may be suggested that a similar inhibition may occur.

Another possibility is that meal termination (*ie*. bout length) is dependent on concentration-dependent chemoreceptor adaptation. Blom (1978) showed that there was a strong correlation between the frequency of impulses from sugar receptors on the mouthparts of *P. brassicae* larvae and the amount of food eaten. The sensory adaptation of mouthpart chemoreceptors to sucrose solutions has been shown in the locust, *Chortoicetes terminifera* (Barton Browne *et al.*, 1975b). On diets of low nutrient content, insects might require a longer period of contact with the food to stimulate chemoreceptors to the level required to initiate adaptation.

Alternatively, instead of monitoring specific nutrients, the insect may be more responsive to a general osmotic effect. The low-C and -P diets are likely to have had lower osmotic pressures than normal diet (although no

measurements were made). A pharyngeal osmoreceptor has been suggested in the cockroach, *Periplaneta americana* (Davey and Treherne, 1963). This osmoreceptor was postulated to influence the rate of crop emptying.

In the case of *Manduca* it would be supposed that such an osmoreceptor would respond to food of lowered osmotic pressure in such a way as to lead to a compensatory increase in bout length and/or bout frequency. *Manduca* does not possess a crop, but an altered rate of passage of food through the foregut might (under the control of putatative osmoreceptors ?) well influence feeding behaviour. Such an interpretation is not supported, however, by the observation that *Manduca* caterpillars fed diets with 2 times and 0.5 times the normal content of added salts did not alter their food intake significantly (K. Bellward, University of Bath, unpublished data).

Several factors have been implicated in the control of the time interval between meals in acridids and blowflies and these include the rate of gut emptying, hormonal and nutrient feedbacks (see reviews by Simpson and Bernays, 1983; Bernays, 1985). The physiological mechanisms involved in the compensation to dietary protein have been examined in locusts (Abisgold and Simpson, 1987). An effect of blood osmolality and amino acid concentration on the time between bouts was discovered. The relationship between nutrient flow and feeding behaviour in *Manduca* larvae is examined in chapter 5.

FIG.4.1. Daily dry weights of food eaten by fifth stadium *Manduca* caterpillars given diets of varying carbohydrate, protein and lipid content. (a) diets with altered digestible carbohydrate content. (b) diets with altered protein content. (c) diets with altered lipid content. Means ± SE (n=10 except where indicated. Smaller sample numbers resulted when some insects ceased to feed and wandered.

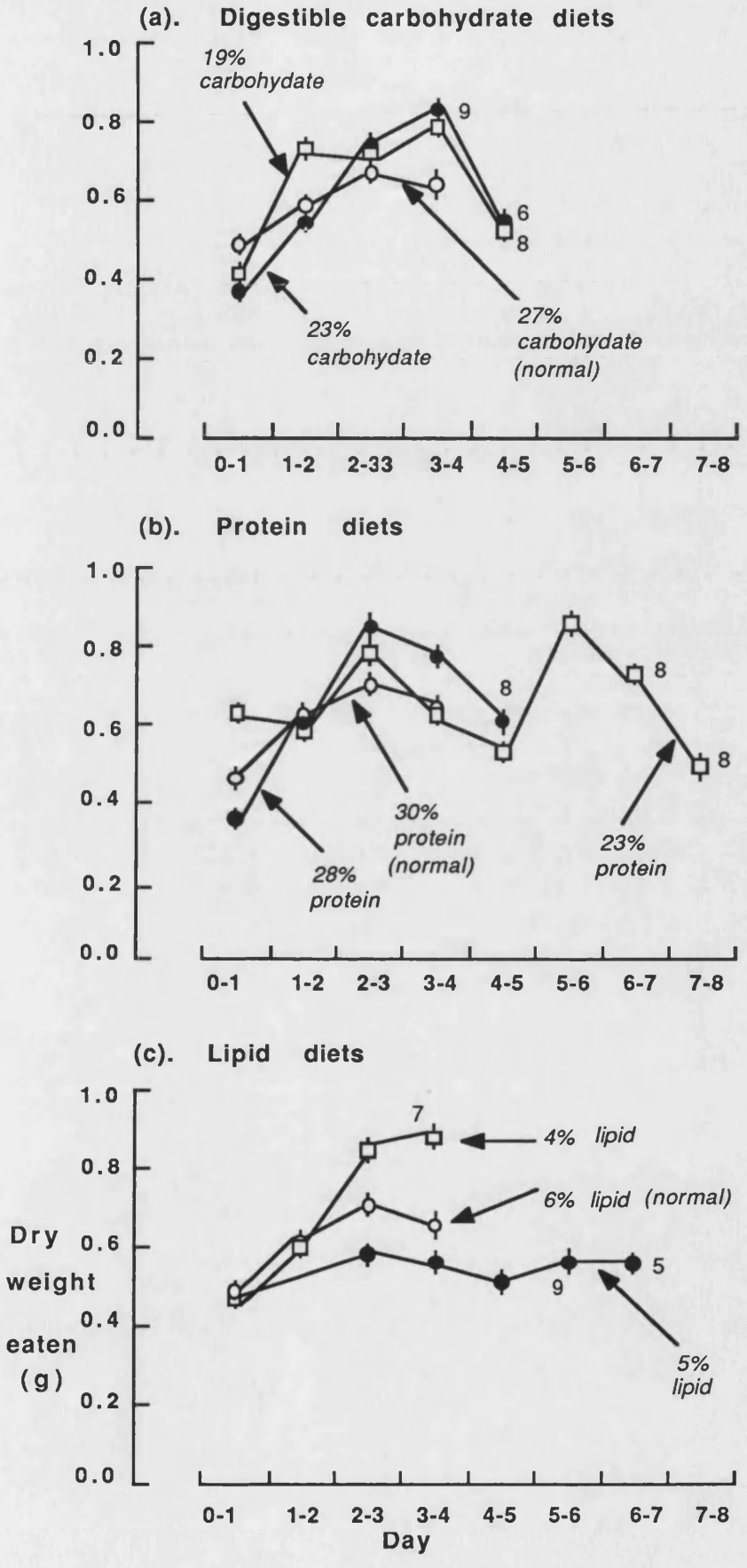
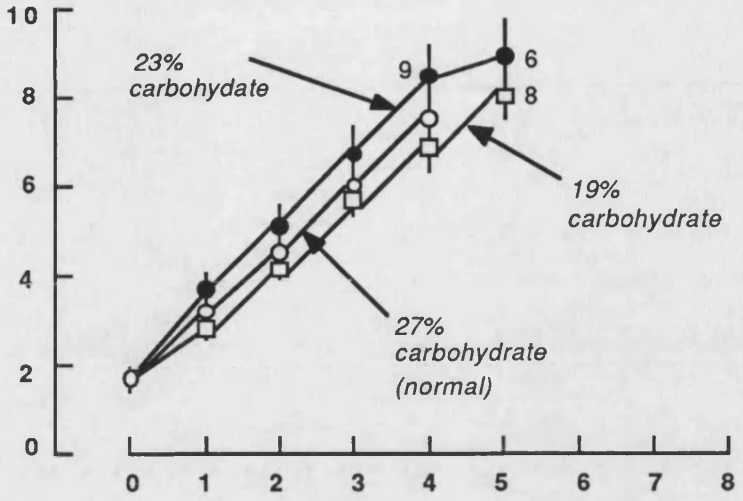
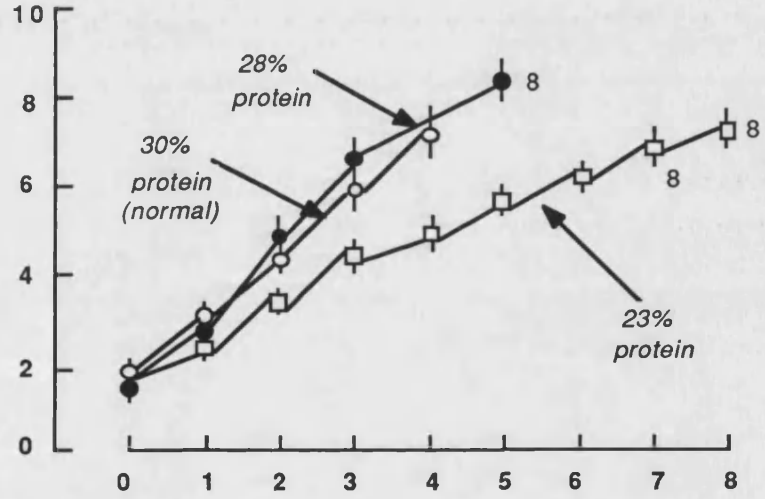

(a). Digestible carbohydrate diets
19% carbohydate
23% carbohydate
27% carbohydate (normal)
9
6
8
(b). Protein diets
28% protein
30% protein (normal)
23% protein
8
8
8
(c). Lipid diets
4% lipid
6% lipid (normal)
5% lipid
7
9
5
Dry weight eaten (g)
1.0
0.8
0.6
0.4
0.2
0.0
0-1
1-2
2-33
2-3
3-4
4-5
5-6
6-7
7-8
Day

FIG.4.2. Growth of fifth stadium *Manduca* caterpillars given artificial diet of varying carbohydrate, protein and lipid content. (a) diets with altered digestible carbohydrate content. (b) diets with altered protein content. (c) diets with altered lipid content. Means $\pm$ SE (n=10 except where indicated. Smaller sample numbers resulted when some insects ceased to feed and wandered).

(a). Digestible carbohydrate diets



(b). Protein diets



(c). Lipid diets

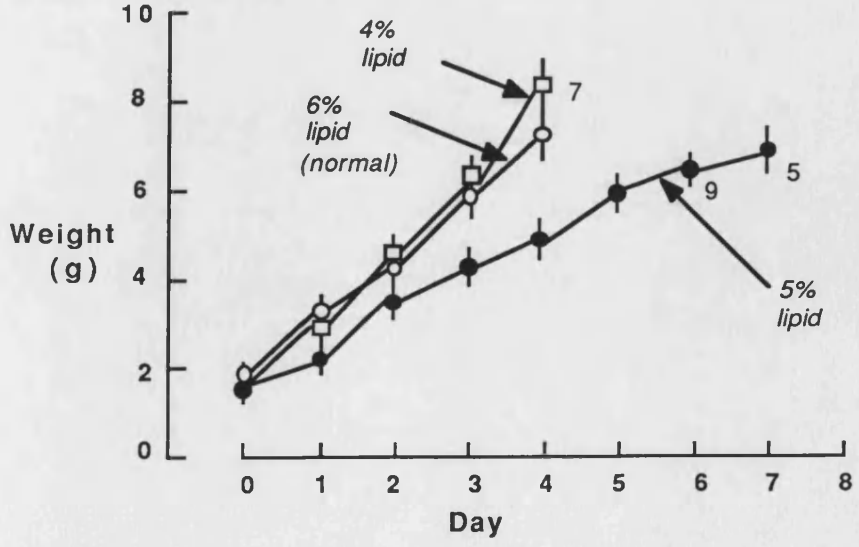


TABLE 4.1 Compositions of experimental artificial diets (means ± SE, n = 5 or 10 as indicated)

(a) digestible carbohydrate diets

	27% digestible carbohydrate content (no added cellulose) (normal diet)	23% digestible carbohydrate content (50% cellulose + 50% sucrose) (medium-C diet)	19% digestible carbohydrate content (100% cellulose + 0% sucrose) (low-C diet)
water (% of fresh weight)	80.33 ± 0.06 (10)	80.37 ± 0.06 (10)	80.27 ± 0.19 (10)
dry matter (% of fresh weight)	19.67 ± 0.06 (10)	19.63 ± 0.06 (10)	19.73 ± 0.19 (10)
digestible carbohydrate (% of dry weight)	27.4 ± 0.4 (5)	23.4 ± 0.2 (5)	19.3 ± 0.5 (5)
protein (% of dry weight)	29.7 ± 0.6 (5)	29.0 ± 2.0 (5)	28.4 ± 1.3 (5)
lipid (% of dry weight)	5.8 ± 0.4 (5)	5.7 ± 0.7 (5)	6.7 ± 0.8 (5)

(b) protein diets

	30% protein content (no added cellulose) (normal diet)	28% protein content (50% cellulose + 50% casein) (medium-P diet)	23% protein content (100% cellulose + 0% casein) (low-P diet)
water (% of fresh weight)	80.33 ± 0.06 (10)	80.47 ± 0.09 (10)	80.25 ± 0.11 (10)
dry matter (% of fresh weight)	19.67 ± 0.06 (10)	19.53 ± 0.09 (10)	19.75 ± 0.11 (10)
digestible carbohydrate (% of dry weight)	27.4 ± 0.4 (5)	28.2 ± 0.4 (5)	26.3 ± 0.8 (5)
protein (% of dry weight)	29.7 ± 0.6 (5)	27.7 ± 0.6 (5)	23.1 ± 0.6 (5)
lipid (% of dry weight)	5.8 ± 0.4 (5)	6.0 ± 0.5 (5)	5.3 ± 0.3 (5)

(c) lipid diets

	6% lipid content (no added cellulose) (normal diet)	5% lipid content (50% cellulose + 50% cholesterol/oils) (medium-L diet)	4% lipid content (100% cellulose + 0% cholesterol/oils) (low-L diet)
water (% of fresh weight)	80.33 ± 0.06 (10)	80.52 ± 0.07 (10)	80.29 ± 0.10 (10)
dry matter (% of fresh weight)	19.67 ± 0.06 (10)	19.48 ± 0.07 (10)	19.71 ± 0.10 (10)
digestible carbohydrate (% of dry weight)	27.4 ± 0.4 (5)	26.0 ± 0.3 (5)	25.9 ± 0.2 (5)
protein (% of dry weight)	29.7 ± 0.6 (5)	29.0 ± 0.6 (5)	29.4 ± 0.9 (5)
lipid (% of dry weight)	5.8 ± 0.4 (5)	4.9 ± 0.4 (5)	3.6 ± 0.2 (5)

TABLE 4.2. Consumption rates (CR) and growth rates (GR) for fifth stadium *Manduca* caterpillars (day 0 to day before wandering) given artificial diets of varying digestible carbohydrate, protein and lipid content (means ± SE, n = 10).

(a) digestible carbohydrate diets

	27% digestible carbohydrate content (no added cellulose) (normal diet)	23% digestible carbohydrate content (50% cellulose + 50% sucrose) (medium-C diet)	19% digestible carbohydrate content (100% cellulose + 0% sucrose) (low-C diet)
dry CR (g/day)	0.60 ± 0.03[a]	0.60 ± 0.04[a]	0.65 ± 0.03[a]
digestible carbohydrate CR (g/day)	0.16 ± 0.01[a]	0.14 ± 0.01[ab]	0.12 ± 0.01[b]
protein CR (g/day)	0.17 ± 0.01[a]	0.16 ± 0.01[a]	0.17 ± 0.01[a]
lipid CR (g/day)	0.13 ± 0.002[a]	0.13 ± 0.002[a]	0.14 ± 0.002[a]
dry GR (g/day)	0.24 ± 0.01[a]	0.29 ± 0.02[a]	0.21 ± 0.01[a]

(b) protein diets

	30% protein content (no added cellulose) (normal diet)	28% protein content (50% cellulose + 50% casein) (medium-P diet)	23% protein content (100% cellulose + 0% casein) (low-P diet)
dry CR (g/day)	0.60 ± 0.03^{a}	0.65 ± 0.02^{a}	0.68 ± 0.02^{a}
digestible carbohydrate CR (g/day)	0.16 ± 0.01^{a}	0.18 ± 0.01^{a}	0.18 ± 0.01^{a}
protein CR (g/day)	0.17 ± 0.01^{a}	0.17 ± 0.01^{a}	0.15 ± 0.005^{a}
lipid CR (g/day)	0.03 ± 0.002^{a}	0.04 ± 0.002^{a}	0.04 ± 0.001^{a}
dry GR (g/day)	0.24 ± 0.01^{a}	0.22 ± 0.01^{a}	0.13 ± 0.01^{b}

(c) lipid diets

	6% lipid content (no added cellulose) (normal diet)	5% lipid content (50% cellulose + 50% cholesterol/oils) (medium-L diet)	4% lipid content (100% cellulose + 0% cholesterol/oils) (low-L diet)
dry CR (g/day)	0.60 ± 0.03^{a}	0.71 ± 0.03^{b}	0.44 ± 0.02^{c}
digestible carbohydrate CR (g/day)	0.16 ± 0.01^{a}	0.18 ± 0.01^{a}	0.12 ± 0.005^{b}
protein CR (g/day)	0.17 ± 0.01^{a}	0.20 ± 0.01^{b}	0.13 ± 0.006^{a}
lipid CR (g/day)	0.03 ± 0.002^{a}	0.03 ± 0.002^{a}	0.02 ± 0.001^{b}
dry GR (g/day)	0.24 ± 0.01^{a}	0.30 ± 0.01^{b}	0.13 ± 0.01^{c}

Significant differences within rows are indicated by different superscripts (one-way ANOVA, 95% confidence intervals based on pooled SD)

* Indicates that significant differences exist between means within the row (Kruskal-Wallis one-way ANOVA by ranks, $p<0.05$). This test was used where an F_{max} test on transformed data showed significant deviation from normality.

TABLE 4.3.The efficiency of food uptake by fifth stadium *Manduca* caterpillars given diets of varying digestible carbohydrate, protein and lipid content (means ± SE, n = 10). AD values for individual nutrients refer only to day 1 insects while AD (dry matter) is calculated throughout the instar.

(a) digestible carbohydrate diets

	27% digestible carbohydrate content (no added cellulose) (normal diet)	23% digestible carbohydrate content (50% cellulose + 50% sucrose) (medium-C diet)	19% digestible carbohydrate content (100% cellulose + 0% sucrose) (low-C diet)
AD (dry matter)	57.35 ± 2.34[a]	52.34 ± 2.67[a]	58.34 ± 0.78[a]
AD (digestible carbohydrate)	84.17 ± 1.20*	50.81 ± 5.35*	64.39 ± 3.13*
AD (nitrogen)	73.44 ± 2.82[a]	67.07 ± 1.13[a]	70.67 ± 0.66[a]
AD (lipid)	64.50 ± 3.81[a]	37.67 ± 5.80[b]	62.33 ± 3.78[a]

(b) protein diets

(b) protein diets

	30% protein content (no added cellulose) (normal diet)	28% protein content (50% cellulose + 50% casein) (medium-P diet)	23% protein content (100% cellulose + 0% casein) (low-P diet)
AD (dry matter)	57.35 ± 2.34^{a}	48.82 ± 1.39^{b}	49.25 ± 1.60^{b}
AD (digestible carbohydrate)	84.17 ± 1.20^{a}	81.14 ± 0.75^{a}	81.13 ± 0.68^{a}
AD (nitrogen)	73.44 ± 2.82^{a}	61.13 ± 1.48^{b}	74.42 ± 1.60^{a}
AD (lipid)	64.50 ± 3.81^{a}	49.33 ± 4.25^{ab}	34.83 ± 7.10^{b}

(c) lipid diets

	6% lipid content (no added cellulose) (normal diet)	5% lipid content (50% cellulose + 50% cholesterol/oils) (medium-L diet)	4% lipid content (100% cellulose + 0% cholesterol/oils) (low-L diet)
AD (dry matter)	57.35 ± 2.34^{a}	54.81 ± 1.02^{a}	55.75 ± 2.00^{a}
AD (digestible carbohydrate)	84.17 ± 1.20^{a}	57.17 ± 3.40^{b}	59.16 ± 2.30^{b}
AD (nitrogen)	73.44 ± 2.82*	49.25 ± 5.21*	67.52 ± 0.87*
AD (lipid)	64.50 ± 3.81^{a}	48.33 ± 3.89^{b}	56.67 ± 2.43^{ab}

Significant differences within rows are indicated by different superscripts (one-way ANOVA, 95% confidence intervals based on pooled SD).

* Indicates that significant differences exist between means within the row (Kruskal-Wallis one-way ANOVA by ranks,$p < 0.05$). This test was used where an F_{max} test on transformed data showed significant deviation from normality.

TABLE 4.4.The efficiency of utilisation of food by fifth stadium *Manduca* caterpillars given diets of varying digestible carbohydrate, protein and lipid content (means ± SE, n = 10).

(a) digestible carbohydrate diets

	27% digestible carbohydrate content (no added cellulose) (normal diet)	23% digestible carbohydrate content (50% cellulose + 50% sucrose) (medium-C diet)	19% digestible carbohydrate content (100% cellulose + 0% sucrose) (low-C diet)
ECI	41.17 ± 2.24*	47.45 ± 3.33*	32.33 ± 0.93*
ECD	71.53 ± 7.93*	95.55 ± 8.88*	57.08 ± 1.59*

(b) protein diets

	30% protein content (no added cellulose) (normal diet)	28% protein content (50% cellulose + 50% casein) (medium-P diet)	23% protein content (100% cellulose + 0% casein) (low-P diet)
ECI	41.17 ± 2.24^a	34.85 ± 0.86^b	19.44 ± 0.61^c
ECD	71.53 ± 7.93^a	72.18 ± 3.48^a	40.28 ± 2.86^b

(c) lipid diets

	6% lipid content (no added cellulose) (normal diet)	5% lipid content (50% cellulose + 50% cholesterol/oils) (medium-L diet)	4% lipid content (100% cellulose + 0% cholesterol/oils) (low-L diet)
ECI	41.17 ± 2.24*	41.99 ± 0.87*	29.20 ± 1.57*

ECD	71.53 ± 7.93*	77.05 ± 2.92*	53.20 ± 3.29*

Significant differences within rows are indicated by different superscripts (one-way ANOVA, 95% confidence intervals based on pooled SD).

* Indicates that significant differences exist between means within the row (Kruskal-Wallis one-way ANOVA by ranks,$p<0.05$). This test was used where an F_{max} test on transformed data showed significant deviation from normality.

TABLE 4.5. Retention time (T_r) of food in the gut of day 1 fifth stadium *Manduca* caterpillars given artificial diets of varying digestible carbohydrate, protein and lipid content (means ± SE, n=10).

(a) digestible carbohydrate diets

	27% digestible carbohydrate content (no added cellulose) (normal diet)	23% digestible carbohydrate content (50% cellulose + 50% sucrose) (medium-C diet)	19% digestible carbohydrate content (100% cellulose + 0% sucrose) (low-C diet)
T_r (h)	7.92 ± 0.70[a]	7.25 ± 0.51[a]	6.40 ± 1.22[a]

(b) protein diets

	30% protein content (no added cellulose) (normal diet)	28% protein content (50% cellulose + 50% casein) (medium-P diet)	23% protein content (100% cellulose + 0% casein) (low-P diet)
T_r (h)	7.92 ± 0.70[a]	6.84 ± 0.28[a]	5.51 ± 0.37[a]

(c) lipid diets

	6% lipid content (no added cellulose) (normal diet)	5% lipid content (50% cellulose + 50% cholesterol/oils) (medium-L diet)	4% lipid content (100% cellulose + 0% cholesterol/oils) (low-L diet)
T_r (h)	7.29 ± 0.70[a]	6.84 ± 0.56[a]	6.48 ± 0.42[a]

Significant differences within rows are indicated by different superscripts (one-way ANOVA, 95% confidence intervals based on pooled SD).

T_I is estimated as dry weight of food in the gut divided by dry weight of food eaten per h.

TABLE 4.6.Parameters of feeding behaviour in day 0 fifth stadium *Manduca* caterpillars given artificial diets of low carbohydrate, protein and lipid content (means ± SE, n=10).

	100% nutrient content (no added cellulose) (normal diet)	19% dig. carb. content (100% cellulose + 0% sucrose) (low-C diet)	23% protein content (100% cellulose + 0% casein) (low-P diet)	4% lipid content (100% cellulose + 0% cholesterol/ oils) (low-L diet)
Percentage of time spent feeding (%)	5.34 ± 0.56^{a}	6.39 ± 0.36^{a}	10.04 ± 0.69^{b}	4.29 ± 0.32^{a}
Bout length (min)	2.51 ± 0.48^{a}	3.65 ± 0.64^{b}	3.24 ± 0.88^{bc}	2.57 ± 0.42^{ac}
Gap length (min)	47.75 ± 10.58^{a}	43.93 ± 11.22^{a}	26.92 ± 6.65^{b}	50.08 ± 12.22^{a}
Bout frequency (h^{-1})	1.10 ± 0.10*	1.05 ± 0.06*	1.76 ± 0.20*	0.99 ± 0.05*
Bout criterion (min)	10.45 ± 1.26^{a}	7.65 ± 1.77^{a}	7.40 ± 1.87^{a}	8.56 ± 1.72^{a}

Significant differences within rows are indicated by different superscripts (one-way ANOVA, 95% confidence intervals based on pooled SD).

* Indicates that significant differences exist between means within the row (Kruskal-Wallis one-way ANOVA by ranks,$p<0.05$). This test was used where an F_{max} test on transformed data showed significant deviation from normality.

Chapter 5

Haemolymph composition and meal initiation in *Manduca sexta* larvae feeding on normal and low-protein diets

Introduction

Manduca caterpillars like locusts (Simpson, 1982) eat discrete meals (chapter 2; Reynolds *et al.*, 1986). Also like locusts (Simpson and Abisgold, 1985), they can alter their feeding behaviour in response to changes in food quality (chapter 3). It is possible that an understanding of how altered food composition leads to changes in meal length and/or frequency may illuminate the factors governing meal initiation and termination under normal circumstances.

Various underlying mechanisms have been proposed to control food intake (reviewed by Dethier, 1976; Simpson and Bernays, 1983). The osmotic pressure of the blood and it's content of various nutrients have been identified as important factors in the control of compensation to dietary protein in locusts (Abisgold and Simpson, 1987). The role of nutrient feedbacks in the control of food intake has been extensively studied in vertebrates (reviewed by Le Magnen, 1985). The aim of the present study was to examine the role of blood nutrients and osmotic pressure in the control of meal initiation in *Manduca* caterpillars feeding on normal and low-protein content diets.

Materials and methods

Rearing conditions and diet compositions

Insects were maintained until the day of ecdysis to the fifth instar under standard conditions (see appendix 1). Newly moulted larvae, weighing between 1.6 and 1.8g, were transferred to normal or low protein (low-P) diet

within 2-3 hours of lights-on (07:00h). The low-P diet was made by substituting all of the casein in the diet with cellulose powder (see chapter 3) to produce a diet containing 23% protein (dry weight) compared with a normal value of 30% protein. Larvae were allowed to feed normally for 4-5 h before samples were collected.

Collection of haemolymph samples

Insects were observed feeding on normal and low-P diets and haemolymph samples were collected at various times after the end of the previous meal. A meal was considered to have begun (S) once the caterpillar had ingested for 15sec and the end of the meal (E) was defined by a minimum of 2 min of feeding followed a non-feeding period of 10min (bout criterion for day 0 insects, see chapter 4). Insects were then removed from the diet and transferred to empty cups for various lengths of time. Faecal pellets were removed from the cups at intervals to prevent coprophagy.

Blood was collected by cutting off the 'horn' and bleeding under gravity (aproximately 250µl collected in 30 sec) into an eppendorf tube (1.5ml) containing a few crystals of phenylthiourea to prevent blackening. Blood samples were immediately frozen on dry ice and used within a few hours or stored at -40°C for 2-3 weeks.

The need to allow a minimum period of 10 min to elapse after the last observed feeding event in order to be sure that the meal had ended meant that it was not possible to collect blood samples at 'E' itself. The earliest samples could only be taken at 'E+10'. The timings given for subsequent samples include the 10 min bout criterion. Thus a sample taken at '1h' is taken at 50 min after the bout criterion has elapsed.

Osmotic pressure of haemolymph

The osmotic pressure of 50µl haemolymph samples was measured using a Roebling depression of freezing point osmometer. The calibration of the instrument was checked before each set of samples using distilled water and 300 mosmolkg^{-1} standards. The results from the osmometer were highly reproducible (300 mosmolkg^{-1} standard, mean ± SE, 299.8 ± 0.3, n = 10).

Chemical compostion of haemolymph from insects fed normal diet

Haemolymph samples were analysed for several nutrients so that changes in their levels with time since a feeding bout could be detected.

Carbohydrates (total reducing sugars) were determined by the anthrone method (Roe, 1955). 10µl of haemolymph was dried under reduced pressure (Speedvac, Savant) in pyrex tubes and then redissolved in 0.2ml H_2SO_4. Tubes were completely covered in aluminium foil, boiled for 10 min and allowed to cool at room temperature for 15 min. 0.15ml 6N NaOH was added after which the tubes were boiled for 10 min. 3.0ml anthrone reagent was added and the tubes boiled for 15 min. Tubes were allowed to cool at room terperature (18-26°C) for 30 min and the optical density (OD) was measured at 620nm using a Cecil (CE373) spectrophotometer. Trehalose standards were taken through the same procedure to produce a standard curve and haemolymph sugar levels were expressed as trehalose equivalents. Anthrone reagent was prepared by heating a mixture of 500 mg anthrone powder and 10g thiourea in 1litre of 66% (v/v) H_2SO_4 at 90°C until all the solid material dissolved.Folowing the procedure of Wyatt and Kalf (1957), the acid-alkali stable carbohydrate may be equated with trehalose.

Protein levels were measured by the folin-phenol method of Lowry *et al.* (1961). The reagents used were: A. 1M NaOH; B. 2% Na_2CO_3; C. 2% sodium tartrate; D. 1% $CuSO_4.5H_2O$ and E. Folin and Ciocalteu's phenol ragent. 0.25ml A, 5.0ml C and 5.0ml D were mixed together (whirlimix, 10sec) and 1.0ml of this mixture (A/B/C/D) was added to 50ml of B. E was diluted 2-fold with distilled water. 10µl of haemolymph, 190µl of distilled water and 200µl of A were boiled for 5min in a capped pyrex tube. After allowing 15min to cool, 2.0ml of A/B/C/D was added to the tube and the contents mixed. 200µl of E was added and the contents again mixed. The tubes were left undisturbed for 30min and the OD read at 750nm. Bovine serum albumen (Sigma fraction V) standards were taken through the same procedure to produce a standard curve. Haemolymph protein levels were expressed as bovine serum albumen equivalents.

The sulpho-phosphovanillin method of Zöllner and Kirsch (1962) was used to determine the total lipid content of haemolymph samples. All tubes

were cleaned with a chloroform methanol (1:1, v/v) mixture prior to use. 25µl of haemolymph was mixed with 250µl of H_2SO_4, boiled in capped pyrex tubes for 10min and cooled for 30min at room temperature. 100µl of the contents of the tube were mixed with 1.0ml vanillin reagent (4:1 concentrated phosphoric acid: 13mM vanillin solution, v/v) and left at room te mperature for 30min. The OD was read at 546nm and cholesterol standards were taken through the same procedure to produce a standard curve. Haemolymph lipid levels were expressed as cholesterol equivalents.

The enzymatic (hexokinase) reduction method (Carroll *et al.*, 1970) was used to measure glucose (*NB.* methods based on glucose oxidase cannot be used for *Manduca* blood because of the presence of interfering substances-pers.comm. R. Ziegler, Universität Berlin). Blood samples were deproteinised by adding 900µl of 10% (w/v) trichloracetic acid (TCA) to 100µl of haemolymph in eppendorf tubes (1.5ml). The tubes were left for 10min at room temperature and were then microcentrifuged (microcentaur, MSE) at 3,300 times g(force of gravity) for 5min and the supernatant removed. The pellet was resuspended in 1.0ml 10% TCA and the deproteinisation procedure was repeated twice. The supernatants were pooled and 2.0ml of water-saturated diethylether added (to remove TCA). The tubes were shaken and the layers allowed to separate. The top layer of diethylether and TCA was removed. This procedure was repeated 4 times and the pooled supernatants dried overnight under reduced pressure (Speedvac, Savant).

A Sigma diagnostics kit (no. 115) was used to measure the glucose content of the samples. 17ml of distilled water and 4.0ml of glucose colour reagent were added to 1vial of glucose enzyme reagent. The vial was inverted to mix and 1.0ml added to the samples. The contents of the tube were mixed by gentle swirling and left at room temperature for 5-10 min. 10ml of 0.1N HCl was then added and the OD read at 520nm. Glucose standards were taken through the same procedure to produce a standard curve. All of the above nutrient determinations were performed on the same blood samples collected for each time point from 5 different insects.

Measurement of free amino acid levels in the haemolymph of insects eating normal and low-protein diets

Haemolymph samples were collected from a different set of insects to the above fed either normal or low-P diets. 25µl of blood was deproteinised according to the method described above for glucose. Samples were resuspended in 200µl of 25mM HCl (Aristar grade) containing 50nM norleucine as an internal standard. Amino acids were separated on an automatic amino acid analyser (Hilger ChromaSpek). Samples were applied to a silica column and eluted under a pH gradient from acid (2.2) to alkali (11.5). 1% ninhydrin was mixed with the eluate and the colour reaction detected at 440nm (proline) and 570nm (other amino acids). Retention times and integrated peak area values (LDC 308 integrator) were produced automatically. A standard cocktail of 21 amino acids was run at the beginning and end of each batch of 5 samples to produce standard retention times and integration values for each amino acid. It proved impossible to reliably distinguish between the amino acids glycine and alanine because of their close retention times and so these were treated together in this study. The osmotic pressure of these blood samples was measured by the method previously mentioned.

Injection experiments

The radioisotope dilution method (Hill *et al.*, 1968) was used to estimate the haemolymph volume of day 0 fifth instar caterpillars. 2.5µCi of [^{3}H]-inulin (Amersham) in 10µl of insect saline (Ephrussi and Beadle, 1936) was injected into the haemocoel between abdominal segments 4 and 5. A preliminary experiment demonstrated that the injected [^{3}H]-inulin reached a steady concentration in the blood after 15min and consequently injected insects were bled at 15min after injection. 100µl of blood was added to 4.0 ml scintillant (Optiphase 'Safe', LKB) and counted using a liquid scintillation counter (Rackbeta 1217, LKB). Quench correction was performed using external standards. Haemolymph volume was calculated using the formula:

$$\text{Haemolymph volume } (\mu l) = \frac{v \times i}{n} - 10$$

where v = volume of blood removed for counting
i = dpm of [^{3}H]-inulin injected (determined by counting a 10µl aliquot)

n = dpm in sample removed

(10 is subtracted because this volume of inulin was injected)

The blood volume was cal culated as 810 ± 40µl (mean ± SE, n = 8) for insects of fresh weight 1.6-1.8g, and this value was used in conjunction with information on blood glucose and free amino acid levels to prepare the solutions for the injection experiments. A glucose solution was prepared so that a 10µl injection of this solution would raise the glucose concentration in the haemolymph from the level at 45min since the last meal to the level after the end of the meal (see Fig.5.3). The amount of glucose injected per insect was 167µg after correction for the 1.2% increase in blood volume due to the injection itself. A solution containing this amount of glucose in 10µl of insect saline (Ephrussi and Beadle, 1936), or a control solution of saline, was injected between abdominal segments 4 and 5 using a 25µl Hamilton syringe. Caterpillars were observed feeding on normal diet before an injection and were removed from the food, quickly injected and left for 1min to recover before being put back on the diet.

A solution containing a mixture of amino acids was prepared so that an injection of this solution would raise the concentration of each amino acid in the haemolymph of normal-fed insects from the level at 45min since the last meal to that observed after the end of the meal (Table 5.1). The amount injected of each amino acid is given in Table 5.2. The injection procedure used was the same as for the glucose injections.

Results

Osmotic pressure and haemolymph nutrients following feeding on normal diet

Changes in blood osmotic pressure over a 24h period since the lst meal are shown in Fig.5.1. A slight elevation during the course of the meal is not statistically significant. The osmotic pressure remained stable during the first hour of food deprivation but a significant fall ($p<0.05$, one-way ANOVA) was seen after 2h without food. The osmotic pressure continued to fall until 6h after feeding when it stabilised and remained constant for at least the next 18h.

The concentrations of haemolymph carbohydrates (trehalose), proteins and lipids over a 24h period since the last meal are shown in Fig.5.2. The carbohydrate content of the blood was significantly less immediately before a meal (S) than it was just after a meal (E+10); the value before feeding was only 0.6 times that measured at E+10. There was no complementary decrease in carbohydrate level during the first hour following the last meal but significant decreases (0.6 times the level at E+10) were seen at both 2h and 6h after feeding. The concentration of lipids in the haemolymph fell significantly (0.6 times the level at E+10) within 0.5h of the last meal and the concentration remained at this level (except for 6h). The lipid content of the blood was not significantly altered immediately before a meal.

In contrast to the carbohydrate level (trehalose) which showed only limited changes with time, glucose showed a rapid and dramatic decrease in concentration (Fig.5.3) within 45min of the last meal (0.3 times the level at the E+10) and this low level was maintained forseveral hours. The steepest decrease in glucose concentration coincides in time with the mean interfeed length (48min) observed in day 0 fifth-instar larvae fed normal food (see chapter 4)

Osmotic pressure and free amino acid levels in the haemolymph of insects on normal and low-protein diets

The osmotic pressure of the blood following feeding on normal and low-P diets is shown in Fig.5.4. The osmotic pressure of the low-P fed insects fluctuated about the normal values but there was no consistent difference (two-way ANOVA,$p>0.05$).

The total concentration of free amino acids in the blood (Fig.5.5) was significantly higher in insects fed the normal compared with the low-P diet (ANOVA, $p<0.05$). The levels of individual amino acids were compared between insects given the differents diets at several points: (i) before the meal; (ii) at E+10; (iii) 30min after the meal (mean interfeed length for day 0 insects fed low-P diet, chapter 4) and (iv) 45min after the meal (mean interfeed length for day 0 insects fed normal diet, chapter 4). The result of this analysis is shown in Table 5.1 and only 2 amino acids out of the 15 present in *Manduca*

blood were found in significantly higher concentrations (two-way ANOVA, $p<0.05$) in the normal diet-fed insects. This difference is not, however, entirely consistent and the levels of both these amino acids are slightly higher at S (see Fig.5.6a and b).Although the total free amino acid concentration was higher in insects fed normal diet this was reflected in higher levels of only 2 amino acids. This result can be partly explained by the increase in total amino acid concentration (possibly from the breakdown of storage protein) at 60 and 75min since the last meal in normal-fed insects (Fig.5.5), since these points were not included in the comparison of individual amino acids (Table 5.1).

Total free amino acid concentration showed significant fluctuations (two-way ANOVA, $p<0.05$) with the time since the last meal (Fig 5.5). The total amino acid concentration in the blood of normal-fed insects increased during the meal (2.2 times S) and showed a decrease in concentration (0.6 times E+10) 45min after the meal. For insects fed the low-P diet, there was also a smaller increase (1.2 times S) during the meal which was followed by a decrease (0.5 times E+10) at 45 min after the meal. 13 out of the 15 amino acids found in *Manduca* blood showed significant decreases in their levels in insects fed normal and low-P diets either at S or the average interfeed lengths compared with the levels at E+10 (Table 5.1).

Injection experiments

Caterpillars deprived of food for 45 min and injected with sufficient glucose to elevate the haemolymph glucose level to the value normally seen at E+10 took 10 ± 1 sec (mean $\pm$ SE, n,15) to begin feeding after being put back on the diet compared with 14 ± 3 sec (mean $\pm$ SE, n=13) for saline injected insects. Insects similarly injected with a solution of amino acids in saline took a similar length of time (17 ± 2 sec, mean $\pm$ SE, n=13) to feed after being replaced on the food. In both treatments, there was no significant effect on the time taken to start feeding (t-test, $p>0.05$).

Discussion

Changes in osmotic pressure and haemolymph nutrients in normal feeding insects

During a meal, the osmotic pressure of *Manduca* haemolymph changes rather little, showing only an insignificant increase of 6 mosmolkg^{-1}, (approximately 2%) followed by a fall to the premeal level. Osmotic pressure continues to fall, however, and is significantly depressed by 2h after feeding, reaching a low level that is maintained from 6-24h after the previous meal. This pattern of change is broadly similar to that found in locusts with osmotic pressure declining within a few hours of the last meal (Bernays and Chapman, 1974a). Fluctuations in osmotic pressure following feeding are likely to be related to changes in one or more haemolymph nutrients. In *Manduca* blood, total lipids showed the largest decrease in concentration of any of the nutrients over the 24h starvation period. Lipids are osmotically inactive and hence are unlikely to contribute to any changes in blood osmotic pressue following feeding. In contrast to the results obtained here, the haemolymph lipid level of waxmoth larvae, *Galleria mellonella*, have been reported to be stable over 48h of starvation (Wlodawer and Wiśniewska, 1965).

Dahlman (1973) studied the effect of long periods of starvation (up to 3 days) on the haemolymph composition of fifth instar *Manduca* larvae. The protein levels of larvae starved for 24h after ecdysis to the fifth instar fluctuated within a similar range (9-18 mgml^{-1}) to the values measured in this study (11-17 mgml^{-1}). Dahlman also found a substantial decrease in trehalose levels over this period (from 2.5 to 6 mgml^{-1}) and decreases in haemolymph trehalose following feeding have been noted in other lepid opterans (Saito, 1963; Mall and Pal, 1982). Despite some significant changes during the day, the concentration of carbohydrate (expressed as trehalose equivalents) measured in this study exhibited no general trend to fall over the 24h period of food deprivation. In agreement with this observation is the work of Siegert (1987) who found that the trehalose level of day 3 *Manduca* caterpillars remained steady over 48h of starvation.

The glucose concentration in the blood decreased dramatically within an hour of the previous meal. Large decreases in the glucose level of *Manduca* larvae deprived of food have been noted previously. For example, after 6h of starvation the glucose level was lowered to 3% of the value at the start of starvation in day0 insects (Dahlman, 1973) and to 20% in day 3 insects (Siegert, 1987). The biochemical mechanisms controlling carbohydrate metabolism during starvation in *Manduca* have been examined and it appears

that the decrease in glucose concentration following feeding acts as a specific stimulus for the enzymatic conversion of glycogen to trehalose (Ziegler, 1985). The level of trehalose is,therefore, regulated but glucose appears to be the measured sugar. In vertebrates, a glucostatic hypothesis of feedng control has been suggested (Mayer, 1955) with the availability of blood glucose being the regulated parameter controlling food intake. A regulatory mechanism based on lipid metabolism and feeding rates has also been postulated in rats (Le Magnen *et al.*, 1973). The decrease in blood glucose and lipid levels found in this study implies their possible function as nutrient feedbacks in the control of feeding initiation in caterpillars fed normal diet.

Amino acid levels in normal and compensating insects

Simpson and Abisgold (1985) showed that locusts compensated for reductions in the level of dietary protein by increasing meal frequency while keeping meal size constant. *Manduca* larvae alter their food intake when offered diets of low-P content by eating larger meals more often (see chapter 4). The physiological basis of the compensation in locusts has been studied (Abisgold and Simpson, 1987) and the interval between meals shown to be influenced by both an osmotic effect and by the levels of free amino acids in the haemolymph. The evidence for a similar mechanism operating in *Manduca* caterpillars is more equivocal.

There was no significant difference in osmotic pressure between blood samples collected from low-P and normal diet-fed insects and the levels of only 2 out of 15 amino acids were lower in insects fed the low-P diet. This result contrasts sharply with the situation in locusts where 11 out of 16 amino acids in the haemolymph were found in lower concentrations in the blood of insects fed diets of reduced protein content (Abisgold and Simpson, 1987). Moreover, the injection of a suitable mixture of these amino acids to raise the haemolymph amino acid concentration of locusts given the reduced protein diet to that of insects fed the normal diet, delayed the time taken to the next meal. There are other reports of a decrease in free amino acid concentration in insects fed diets of reduced protein content (Chen and Hadorn ,1955; Strong, 1964). Why, therefore, were there not more decreases in the levels of amino acids in the haemolymph of *Manduca* caterpillars fed the low-P diet?

A possible explanation might be that the degree of protein dilution in this study was not as extreme as that used by Abisgold and Simpson. In the locust study, the low-P diet contained only 50% of the normal protein content compared with a low-P value of 78% of normal in this study. Despite this smaller difference the protein content, *Manduca* caterpillars still compensated significantly for reduced dietary protein (see chapter 4).

It has been suggested (chapter 4) that caterpillars might be more sensitive to protein deficiency than acridids because of their higher growth rates and therefore higher protein requirements. If caterpillars were highly sensitive to the protein content of their food, then a decrease in the concentration of even a few amino acids might be sufficient to induce compensation. Alternatively, glutamine and methionine may represent highly specific stimuli for an increase in meal frequency. These amino acids could act directly on centres associated with food intake control or by altering the output from the CNS to such centres. Recently, Simpson and Abisgold (1988) demonstrated that amino acids can have a direct effect on the sensitivity of mouthpart sensilla in locusts.

Glucose and amino acid injections

In addition to providing information on possible nutrient feedbacks involved in the compensation to dietary protein, the amino acid analysis of caterpillar haemolymph also suggested the possible involvement of amino acid levels in the control of interfeed length in normal feeding caterpillars. There was a significant decrease in amino acid concentration 45min after the last meal in the blood of insects fed both normal and low-P diets. A similarly timed but larger fall in the glucose level of insects eating normal diet has also been mentioned. Injections of these nutrients, designed to raise their concentrations back to the level before the decrease, did not affect the time taken to the next meal in insects given normal diet. This result suggests either that these nutrients are not important in the control of meal initiation or that the effect of the treatments was diminished by other factors associated with the injections.

There are some important differences between the behaviour of locusts and caterpillars during the interfeed period. Locusts roost on perches away from the food source while caterpillars remain in constant contact with their food. To inject caterpillars, they must first be removed from the diet, injected and then replaced and caterpillars may gain sensory information from sensilla on the tarsi (Chapman, 1982) while in contact with the food. Reinecke *et al.*(1980) noted the sensitivity of fifth instar *Manduca* larvae to external disturbances. It is possible that the physical disturbance caused by being handled during the injection and loss of chemoreceptor input might raise the level of some 'excitatory state' above a certain threshold required to automatically initiate feeding once the insect is replaced on the food. The inhibitory effects of specific nutrients on the length of time to the next meal might thus be overriden. An attempt was made to circumvent this possible problem by implanting a cannula into the haemocoel (short length of polythene tubing attached to a fine dentists needle). This was used to allow the introduction of glucose solutions directly into the haemolymph while the insect fed on the diet. Unfortunately, this arrangement seriously interrupted normal meal frequency (0.2 times normal) and could not be used as a reliable alternative to injections.

Bernays (1980) has also studied the effect of altering the haemolymph composition on the probability of initiating feeding in locusts. Corpora cardiaca extracts injected into the haemolymph reduced the post-prandial rest and consequently the likelihood of feeding. Artificially filling the crop with agar produced a similar effect. In contrast, the injection of several nutrients (trehalose, glucose, proline and glycine) had no effect on the rest period. It is clear that several factors other than nutrients may affect the likelihood of feeding in caterpillars and the role of gut emptying is examined in chapter 6.

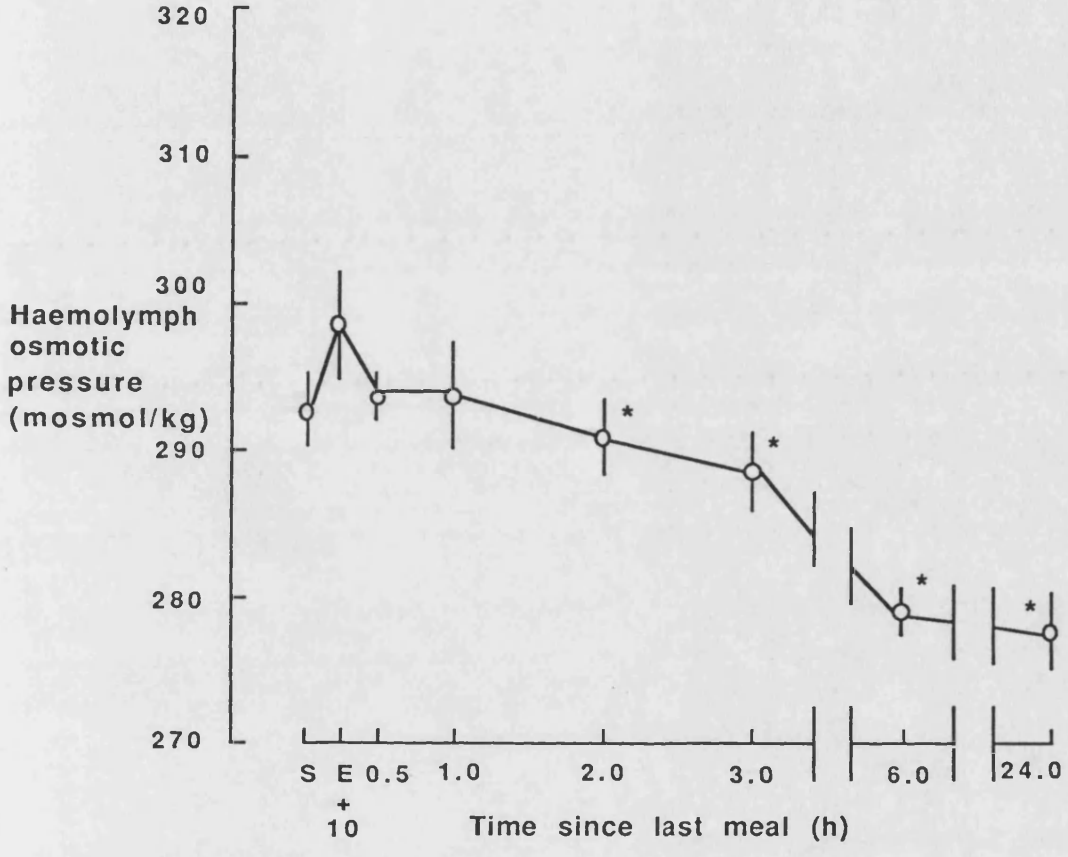


FIG.5.1. The change in haemolymph osmotic pressure with time since the last meal for day 0 fifth stadium larvae fed normal diet. Means ± SE (n=10 per point). Different groups of insects were used for each time point.
* Denotes a significant difference (one-way ANOVA, 95% confidence intervals based on pooled SD) between the indicated point and the value at the end of the meal ('E+10'). 'S' represents the start of the meal.

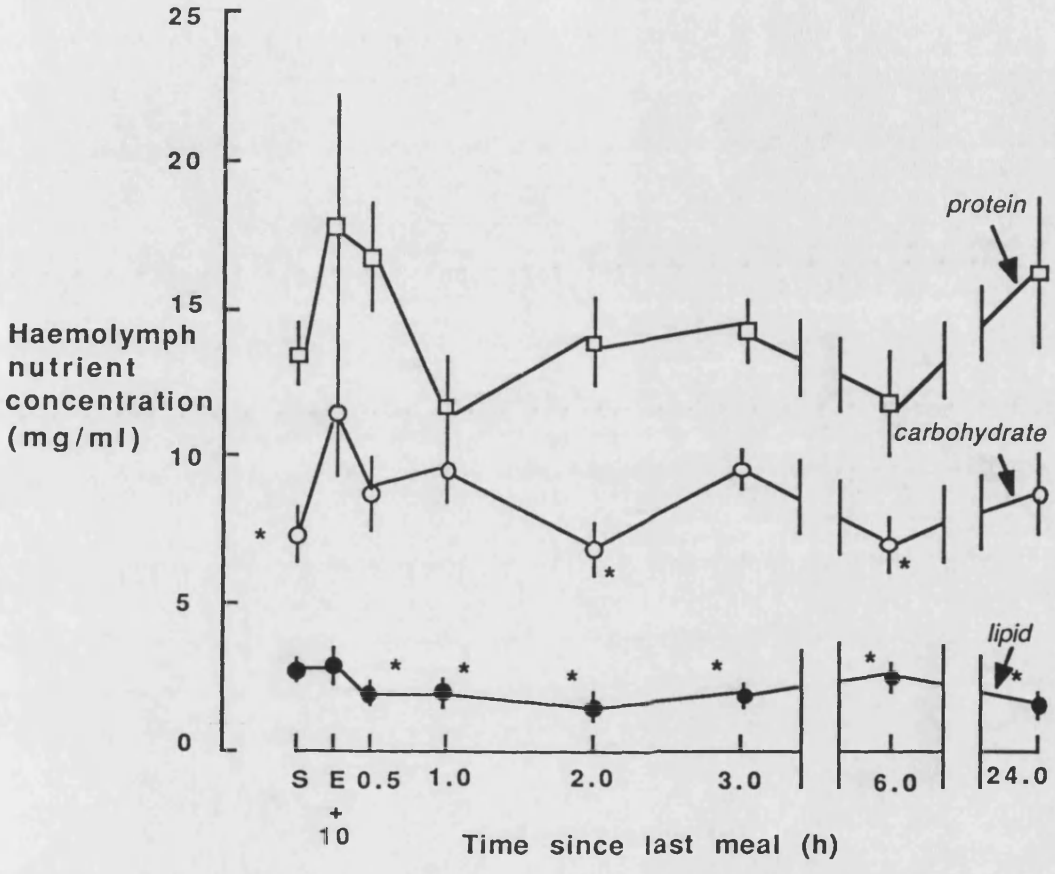


FIG.5.2. The change in haemolymph protein, carbohydrate and lipid levels with time since the last meal for day 0 fifth stadium Manduca larvae fed normal diet. Means ± SE (n=5 per point). Different groups of insects were used for each time point.

* Denotes a significant difference (one-way ANOVA, 95% confidence intervals based on pooled SD) between the indicated point and the value at the end of the meal ('E+10'). 'S' represents the start of the meal.

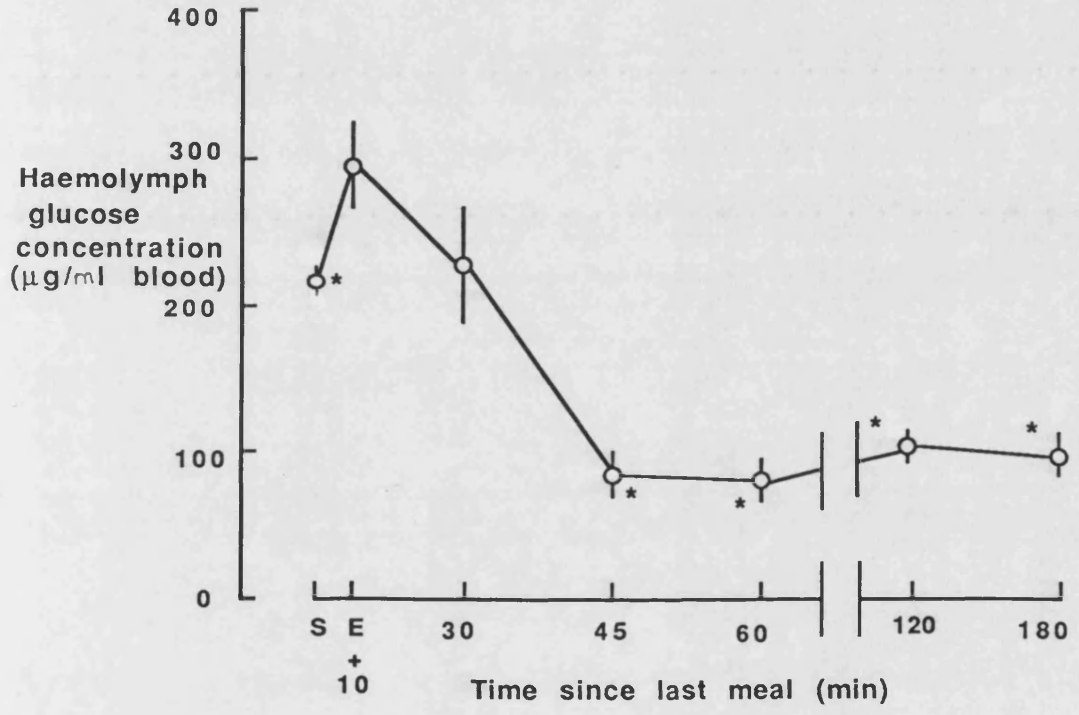


FIG.5.3. The change in haemolymph glucose concentration with time since the last meal for day 0 fifth stadium Manduca larvae fed normal diet. Means ± SE (n=5 per point). Different groups of insects were used for each time point.

* Denotes a significant difference (one-way ANOVA, 95% confidence intervals based on pooled SD) between the indicated point and the value at the end of the meal ('E+10'). 'S' represents the start of the meal.

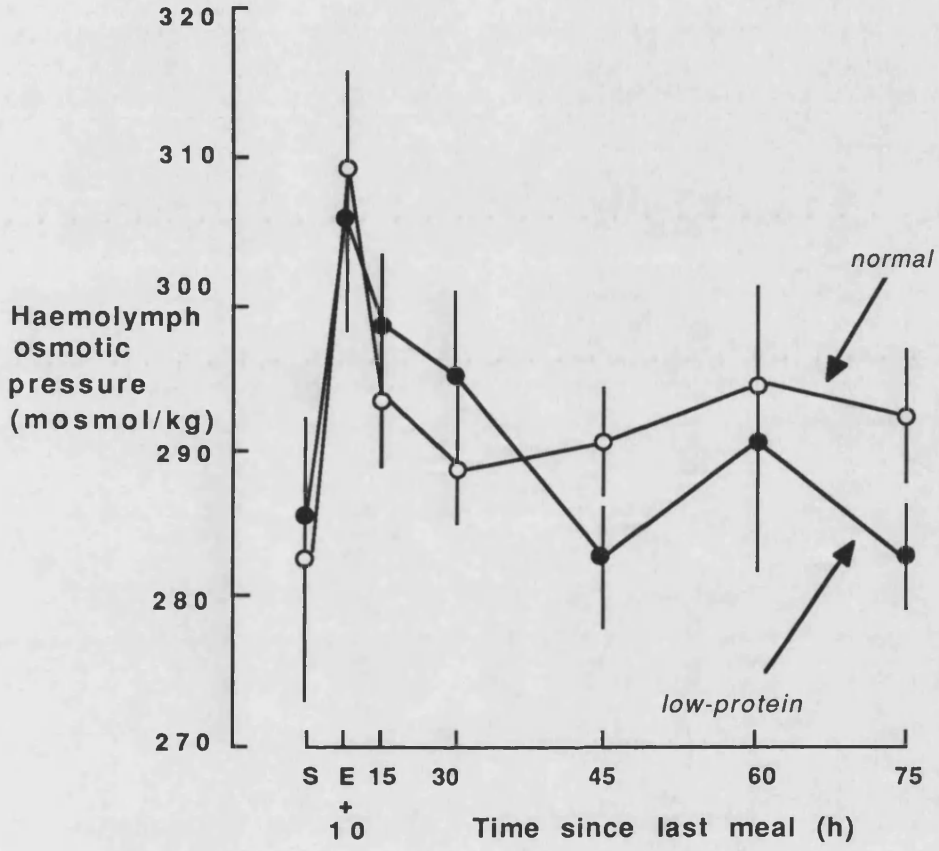


FIG.5.4. The change in haemolymph osmotic pressure with time since the last meal for day 0 fifth stadium Manduca larvae fed normal or low-protein diet. Means + SE (n=5 per point). Different groups of insects were used for each time point. Two-way ANOVA showed: (i) no significant difference between normal and low-protein diets as a main effect or as an interaction ($F=0.46$, df 1,56, $p>0.05$; and $F=0.13$, df 1,6, $p>0.05$); and (ii) no significant difference with time since the last meal as a main effect or as an interaction ($F=2.02$, df 6,56, $p>0.05$; and $F=0.57$, df 6,6, $p>0.05$). The end of the meal is indicated by 'E+10' and 'S' represents the start of the meal.

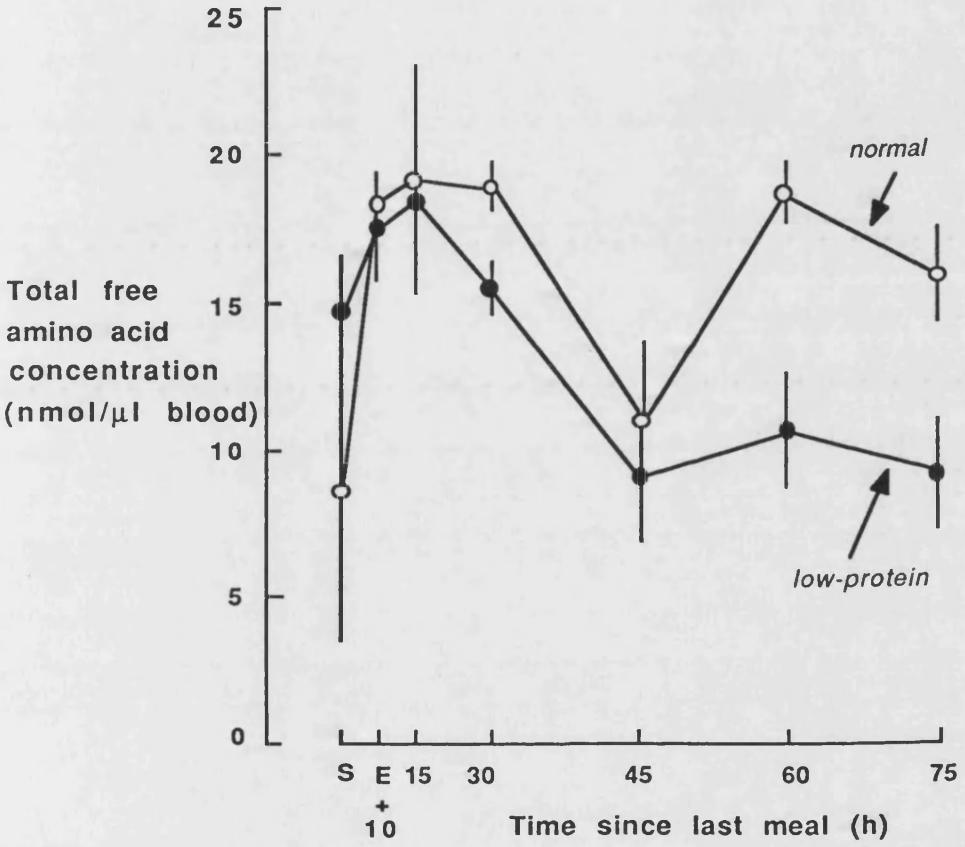


FIG.5.5. The change in total free amino acid concentration with time since the last meal for day 0 fifth stadium Manduca larvae fed normal or low-protein diets. Means ± SE (n=5 per point). Different groups of insects were used for each time point. Two-way ANOVA showed: (i) significant difference between normal and low-protein diets as a main effect ($F=4.50$, df 1, 56, $p<0.05$); and (ii) a significant difference in amino acid concentration with time since the last meal as a main effect ($F=2.33$, df 6, 56, $p<0.05$). The end of the meal is indicated by 'E+10' and 'S' represents the start of the meal.

FIG.5.6. The change in (a) glutamine and (b) methionine concentration since the last meal for day 0 fifth stadium larvae fed normal or low-protein diet. Means ± SE (n=5 per point). Different groups of insects were used for each time point. The end of the meal is indicated by 'E+10' and 'S' represents the start of the meal.

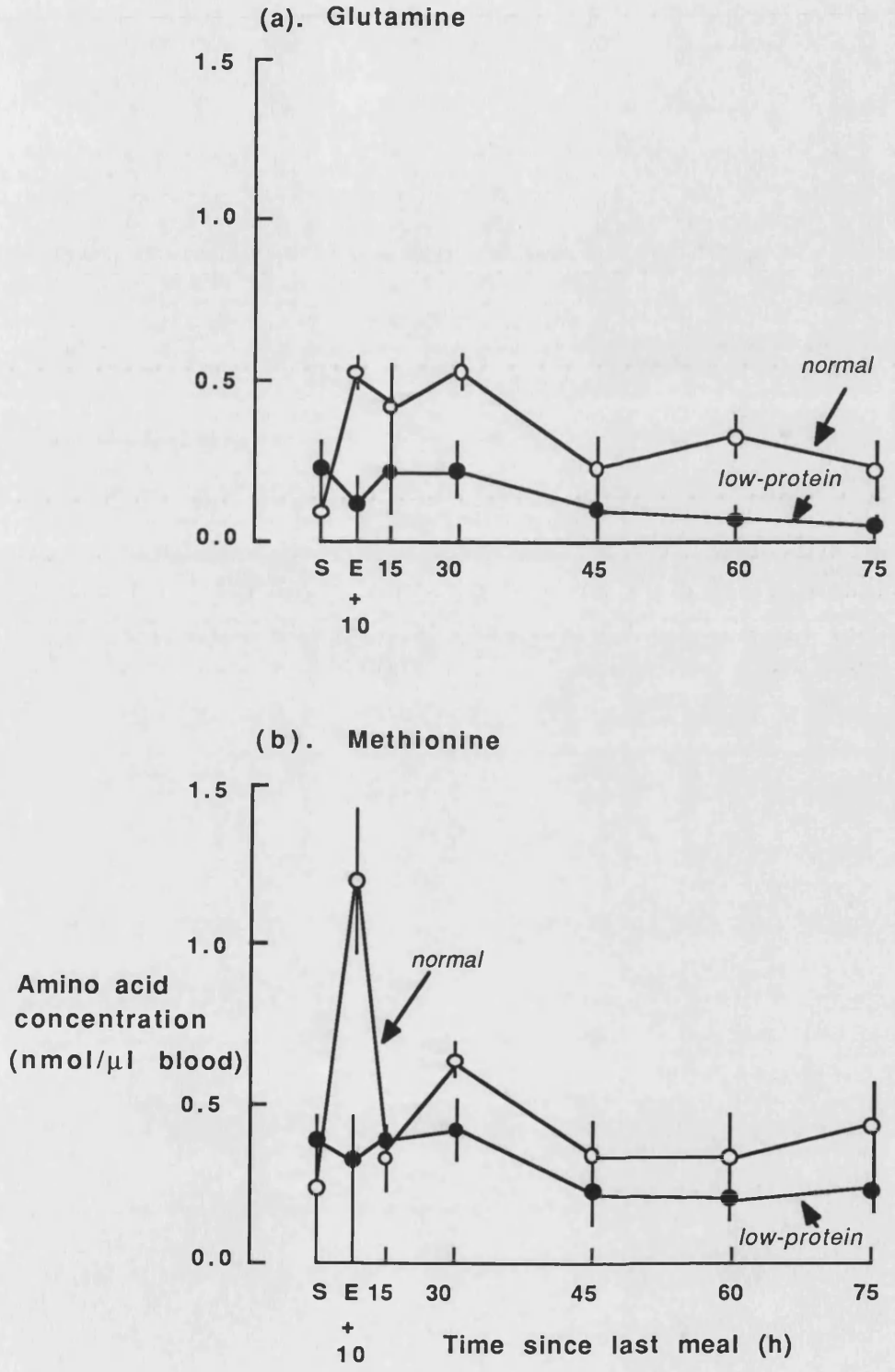

(a). Glutamine
1.5
1.0
0.5
0.0
normal
low-protein
S
E
+
10
15
30
45
60
75
(b). Methionine
Amino acid
concentration
(nmol/μl blood)
Time since last meal (h)

TABLE 5.1. The amino acid concentrations of blood samples collected from day 0 fifth stadium *Manduca* caterpillars at various times after feeding on normal and low-protein diets. Means, n=5.

Amino acid concentration (nmol μl^{-1})

	Insects fed normal diet				Insects fed low-P diet				Significant differences	
	Time since last meal								between insects on	between the a.a. conc.
Amino acid	S	E+10	30	45	S	E+10	30	45	normal & low-P diets	before & after a meal
Aspartic acid	-	0.7	0.6	0.06	0.5	-	0.2	-	NS	*
Threonine	1.4	1.4	2.0	1.3	1.4	2.5	1.6	1.3	NS	*
Serine	2.4	5.1	6.3	3.8	4.3	4.4	5.8	3.4	NS	*
Glutamine	0.07	0.5	0.5	0.2	0.2	0.09	0.2	0.07	*	*
Proline	0.05	0.07	0.1	0.08	0.09	0.08	0.08	0.08	NS	NS
Glycine/Alanine	1.6	3.6	4.0	1.6	2.8	4.3	3.0	1.4	NS	*
Valine	0.3	1.0	0.5	0.4	0.5	0.6	0.4	0.3	NS	*
Methionine	0.2	1.2	0.6	0.3	0.4	0.3	0.4	0.2	*	*
Isoleucine	0.2	0.08	0.03	0.09	0.1	0.2	0.2	0.2	NS	NS
Leucine	0.4	0.3	0.3	0.2	0.4	0.6	0.4	0.02	NS	*
Tyrosine	0.06	0.5	0.3	0.3	0.3	0.8	0.3	0.2	NS	*
Phenylalanine	0.04	0.1	0.03	-	0.04	0.03	0.07	-	NS	*
Histidine	0.9	1.9	1.9	1.2	2.0	1.7	2.1	1.3	NS	*
Lysine	0.6	1.2	0.8	0.6	0.9	0.9	0.8	0.4	NS	*
Arginine	0.2	0.4	0.3	0.2	0.6	0.6	0.3	0.2	NS	*

* Indicates significant differences (two-way ANOVA,$p<0.05$)

NS indicates no significant differences

a.a. is an abbreviation for amino acid

S denotes the start of a meal E+10 denotes the end of a meal

TABLE 5.2. The amounts of amino acids injected into the haemolymph of day 0 fifth stadium *Manduca* caterpillars (means, n=5).

Amino acid	Amount of amino acid (nmol)
Aspartic acid	405
Threonine	81
Serine	1053
Glutamine	243
Proline	-
Glycine/Alanine	1620
Valine	486
Methionine	729
Isoleucine	-
Leucine	81
Tyrosine	162
Phenylalanine	81
Histidine	567
Lysine	486
Arginine	162

The amount of each amino acid was calculated as that required to raise the level found 45min after a bout in the haemolymph of normal-fed insects to that found at the end of a meal. Dashed lines (-) denote those amino acids for which either no change or an increase in concentration occurs with time since a meal.

Chapter 6

Physiological mechanisms underlying the control of normal food intake in *Manduca sexta* larvae

Introduction

It is likely that insects require information about physiological variables which change with time both during and after a meal in order to exert control over meal size and frequency. One such variable that must change as a consequence of feeding is the degree of fullness of the gut. Ingested food is passed along the gut following a meal as it is digested and absorbed. It has been suggested that in locusts changes in the volume of the gut can be detected by stretch receptors and that sensory input from these receptors influences the amount of food eaten (Bernays and Chapman, 1973; Simpson, 1983).

In blowflies, volumetric feedback from the body wall has also been demonstrated to play a role in regulating food intake (Dethier, 1976). The effect appears specific to protein feeding. Belzer (1979) showed that this negative feedback limited the size of protein meals taken by female flies. Because the volume of mature eggs in the abdomen contributes to this feedback, protein feeding is reduced when the fly is gravid with eggs, and automatically enhanced when the fly oviposits. Belzer suggested that this was adaptive in providing protein for the next cycle of ovarian development. Some indirect evidence for the volumetric control of feeding in *Manduca* caterpillars has already been described (see chapter 2; Reynolds *et al.*, 1986). The source of the volumetric sensory feedback that might mediate this is unknown.

In addition, changes in factors associated with the insect's blood have been

shown to influence the likelihood of feeding. For example, hormones may be released which decrease the sensitivity of mouthpart receptors in locusts (Bernays *et al.*, 1972a) and increase the rate of gut emptying (Cazal, 1969). Haemolymph osmotic pressure can change during feeding and may contribute to the control of meal size. Bernays and Chapman (1974a) showed that injections of trehalose, sodium chloride, proline, glycine, sorbose, inulin or a mixture of solutes all decreased the size of the next meal taken by locusts. They concluded that this was an osmotically mediated effect, but noted that the magnitude of normally occurring changes in osmotic pressure during a meal were probably insufficient to affect meal size.

More recently Abisgold and Simpson (1987) showed that xylose injections (used as an osmotic control for amino acid injections) increased the time to the next meal, but xylose was much less effective than were the amino acids. These authors concluded that osmolality affected meal frequency but not meal size (Simpson and Abisgold, 1985; Abisgold and Simpson, 1987). Thus the importance of osmotic effects in locusts remains unclear. Increased osmotic pressure has also been reported to reduce the rate of gut emptying in blowflies (Thomson and Holling, 1977).

Variations in the soluble nutrient composition and osmotic pressure of *Manduca* haemolymph following feeding have been observed previously (see chapter 5). An attempt is made in this study to evaluate the relative importance of these changes in blood composition and of changes in gut volume in the control of normal feeding behaviour in *Manduca* caterpillars.

Materials and methods

Measurement of meal size and gut emptying at intervals following feeding

Insects were reared under standard conditions (see appendix 1) until day 0 of the fifth instar when they were removed and deprived of food for various times according to the procedure described in chapter 4. Only insects weighing 1.6-1.8g were used in this study and the mean weight of each group of insects (*i.e.* individual time points or injections) was adjusted to 1.68 $\pm$ 0.02g (mean $\pm$ 95% confidence interval, n=28). The size of the first meal following food deprivation was measured by weighing the insect to the nearest 1mg before the

meal and then again at the end of the meal. The latter value was corrected for any defaecation that occured during the meal by adding the weight of the faecal pellet.

In chapter 4, a minimum bout length of 2min followed by a period of 10min without feeding was used to define a meal in day 0 fifth instar caterpillars. In this study, it was possible that changes in meal size might occur as a result of the injections and so the previous definition of a minimum period of feeding was no longer appropriate. Instead, insects were allowed to feed on the diet for 30min and any weight increases after this period of at least 5mg were classed as representing 'meals'. Values of below 5mg were considered to represent a failure to feed in the allotted time period. A meal size of 5mg was chosen as the criterion for feeding because any values less than this were considered to be within the experimental error associated with weighing the insects. The values for times to the next meal and meal sizes shown for various treatments in Figs. 6.1, 6.3 and 6.4 refer only to measurements of 5mg or more and do not include insects which failed to feed within 30min of the injection.

The rate of gut emptying was estimated by rapidly anaesthetising insects (exposed to CO_2 gas in a beaker for 30sec) and dissecting out the whole gut at various times after the meal. The gut was separated into the foregut (pharynx and oesophagus), midgut (including malpighian tubules) and hindgut (pylorus, ileum, colon and rectum). The different portions of the gut were blotted dry on tissue paper for 2-3min to remove excess haemolymph, and weighed to an accuracy of 0.1mg.

Injections of a paraffin mixture

Standard feeding parameters were recorded for each type of injection experiment. These included: (i) the time taken to start feeding after being put back on the diet; (ii) the size of the meal eaten; and (iii) the number of insects feeding within 30min of being replaced on the diet.

A mixture of paraffin oil: paraffin wax (2:1, v/v) was injected into the fore- and midgut of *Manduca* caterpillars in order to simulate the volume occupied by ingested food in the gut (Roessingh and Simpson, 1984). Using

information obtained from measurements of the sizes of meals and the degree of gut emptying following various periods of food deprivation, appropriate volumes and times of injections were selected.

Insects were found to eat meals of 32-36mg (Fig6.1) when deprived of food for periods of up to 45min, the average interfeed length for day 0 insects (see chapter 4). Injections of 40 and 80µl of the paraffin mixture approximated the volume occuppied in the gut by 1 and 2 average sized meals, respectively.

These volumes were injected into the foregut at the approximate interval (*i.e.* 45min after feeding) by inserting the needle of a 100µl Unimetrics syringe (gauge 28) through the mouth and into the foregut. A preliminary study using a coloured paraffin mixture (Sudan red dye), revealed that it was necessary to insert the syringe needle approximately 0.5cm into the mouth to ensure that the injected material entered and remained in the foregut and not the midgut. Following the injection, insects were allowed 2min to recover before being replaced on the diet. For control insects, the needle was inserted into the mouth and left in place for the same length of time as the experimental insects but the paraffin mixture was not injected.

A range of injection volumes (0-150µl) which covered the changes in meal size and gut weights observed as a result of food deprivation were used in the midgut injections. The injections were made into the midgut through the body wall between abdominal segments 4 and 5 at 2h after the previous meal. A 1.0ml disposable syringe (Gillette) fitted with a hypodermic needle (Terumo, gauge 27) was used to perform the injections. Preliminary studies with the dyed paraffin mixture revealed that the injected material was deposited in the anterior half of the midgut.

The use of the paraffin oil/wax mixture in these experiments ensured that the gut volume was increased by the volume actually injected. This is an important point, since water might be absorbed from an aqueous solution, reducing the volumetric effect of such an injection (Roessingh and Simpson, 1984).

Injections of a crude diet extract

In order to test for the effects of nutrients on the time to the next meal and meal size, an extract of soluble nutients from the artificial diet eaten by *Manduca* caterpillars (see appendix 1) was prepared and injected into either the haemocoel or the midgut. The diet extract was prepared by mixing the normal quantity of premix (336g) with twice the normal volume (700ml) of boiling distilled water. This larger volume (*i.e.* 1400ml) of water was used to increase the amount of nutrients extracted from the premix and 5.0ml of this extract was freeze-dried overnight (Speedvac,Savant). Assuming the above procedure extracted all of the soluble nutrients in the premix, then the residue would contain approximatley 1g of nutrients. For each block of diet, the ratio of nutrients to non-nutrients (water and agar) was 1:5 and so the nutrient residue was resuspended in 5.0ml of insect saline (Ephrussi and Beadle,1936). Other nutrients (corn oil, linseed oil, ascorbic acid and Vanderzant vitamin mixture) were added in proportion to the amount of nutrient extracted (approximately 1g) and the normal nutrient content of the premix (336g). Therefore, the amounts of these other nutrients added to the extract solution were 1/336th of the quantities listed in appendix 1.

The meal size of insects starved for 2h was found to be 91 $\pm$ 5mg (mean $\pm$ SE, see Fig.5.2) and insects were injected with 45µl (approximately 0.5 times the nutrient content of the meal) and 90µl (approximately 1.0 times) of the diet extract. The injections were performed between abdominal segments 4 and 5 into the haemocoel and midgut using a 100µl Unimetrics syringe. The control insects received 90µl of insect saline.

Injections of xylose

The increase in osmotic pressure produced as a result of the diet extract injections was measured using a Roebling osmometer (see chapter 4). Blood and midgut samples were collected 5min after the injection of 90µl of diet extract into 2h-starved insects. The haemolymph osmotic pressure increased by 44 mosmolkg^{-1} (approximately a 14% increase) following the injection and the midgut osmotic pressure rose by 45 mosmol kg^{-1} (approximately a 16% increase). Xylose (a non-nutrient sugar not utilised by insects) was used as an osmotic control for this increase. A solution of xylose in insect saline was adjusted to the same osmotic pressure as the diet extract (718 $\pm$ 1 mosmolkg^{-1},

mean ± SE, n=5); 90µl of this solution were injected into either the haemocoel or the midgut. The injection of solutions of xylose and diet extract into the haemocoel and midgut not only increa sed the nutrient content and osmotic pressure of these body components but also increased their volumes. From the previously measured blood volume of day 0 insects (see chapter 5), it was calculated that a 90µl injection would increase the blood volume by approximately 11%. A rough estimate of midgut volume based on midgut weight (Fig.6.2b) suggests that a 90µl injection would increase midgut volume by 38%. As a control for the volumetric effects of the injections, 90µl of insect saline solution was injected into either the haemocoel or the midgut of insects previously starved for 2h.

Recurrent nerve operations

Day 0 fifth instar caterpillars were anaesthetised with CO_2 by placing them in a beaker containing dry-ice for 1min. The head capsule of the insect was then inserted through a hole in the lid of the beaker and positioned so that it rested on the surface of the lid, while the rest of the body remained in the beaker under continuous CO_2- anaesthesia. Working under a binocular microscope with a cold light source, a piece of cuticle was removed from the clypeus of the head capsule using a fine scalpel to expose the frontal ganglion and recurrent nerve. The recurrent nerve was carefully cut using microscissors between the frontal and hypocerebral ganglia. It was easy to see when the recurrent nerve had been successfully severed. Neither the frontal nerves nor the frontal ganglion itself were damaged during this procedure. Following the operation, the detached piece of cuticle was sealed back in position in the clypeus using a small amount of molten paraffin wax. Insects were allowed 2h without food to recover after the operation and the size of the first meal after this period was measured. Sham-operated insects underwent the same treatment as recurrent nerve-cut insects except that instead of severing the nerve, it was gently touched with the tip of the microscissors.

Results

Meal size and gut emptying

Meal sizes of *Manduca* caterpillars under normal and food-deprived

conditions are shown in Fig.6.1. Insects ate a meal of between 32 and 36mg in size when feeding with constant access to diet and when deprived of food for periods up to 45min (the average intermeal interval of day 0 insects, see chapter 4). After 60min of food deprivation, the size of the meal eaten increased significantly (ANOVA, $p<0.05$) to 2.0 times the normal value (*i.e.* with no deprivation) and a further increase was observed at 120min (2.7 times normal). A limit to this increase was reached at 180min without food when the meal size stabilised at around 90mg. It was possible that the caterpillars might also alter their meal frequency in response to imposed periods of starvation. This idea was investigated in a preliminary study by filming the feeding behaviour (see chapter 3) of caterpillars starved for 0 and 2h and then returned to normal diet. The gap between the first and second meals taken after being returned to the food was the same (t-test, $p>0.05$) for undeprived (53.7 ± 3.2min, mean $\pm$ SE, n=10) and 2h-starved insects (50.1 ± 2.6min, mean $\pm$ SE, n=10). Thus, although caterpillars that have been deprived of food for longer than 45min increase the size of their first meal they do not increase the frequency of meal-taking.

Fig.6.2 shows the fresh weights of fore-, mid- and hindguts at various times after the previous meal. As might be expected the fresh weight of the foregut increased significantly (ANOVA, $p < 0.05$) to 2.6 times the value before the meal (S) as the meal was ingested. As previously explained (chapter 4), the first time point at which it was possible to be confident that a meal had actually ended was 10min after the last observed ingestion behaviour (E+10). At this time the foregut weighed about 12mg, or about one third of the weight of the food ingested during the meal (32-36g). Since the weight of the empty foregut was about 4mg, the contents must weigh about 8mg. This means that about one quarter of the food ingested during a meal is still present in the foregut 10min after the meal has ended. Subsequently, the weight of the foregut decreased, finally reaching a constant value at around 45min after the meal, by which time the foregut had emptied into the midgut. A 32% increase in the weight of the midgut (0.3 times S) was observed during the meal, presumably as a result of food entering from the foregut. The midgut weight fell significantly (0.8 times E+10) at 45min after the last meal and remained at this level for the next few hours.In contrast to the fore- and midgut, no indication of a weight increase was observed in the hindgut during the meal and, in addition, there were no significant variations following the meal.

Paraffin injections

The effects on feeding of injecting a mixture of liquid paraffin and paraffin wax into either the foregut or the midgut are summarised in Fig.6.3. Some of the injected insects failed to reinitiate feeding within 30min (Fig.6.3a). There did not seem to be any pattern to this response, which may have been due to physical trauma. Since all the other insects fed almost immediately, the data on meal sizes and times taken to initiate feeding may be taken to represent the responses of unharmed animals.

Injections of the mixture into the foregut caused changes in meal size but not the time taken to the next meal. Surprisingly, the injection of 40µl of the mixture (equivalent to the approximate volume of 1 meal) into the foregut significantly increased meal size (2.0 times control). An increase in the time taken to resume feeding after the injection (1.6 times control) was not statistically significant (ANOVA, $p > 0.05$).Doubling the volume injected to 80µl resulted in a smaller and non-significant increase in meal size (1.6 times control). The time taken to resume feeding after an 80µl injection was the same as in the injected controls. There was no significant alteration in either of the above feeding parameters when 50,100 or 150µl of the paraffin mixture were injected into the midgut.

Diet injections

Changes occurred in both the time taken to start feeding and meal size in response to diet extract injections into the haemocoel and midgut (Fig.6.4). The injection into the haemocoel of 90µl of the extract (approximately equivalent to the nutrient content of 1 meal) caused a significant increase in the time taken to start feeding (1.7 times saline control) and a significant decrease in the size of the meal (0.2 times saline control). Infact, the effect of this injection was sufficient to inhibit 58% (7 out of 12) of the injected insects from feeding within 30min of being placed back on the food. The effect of the diet extract on meal size appeared to be dose-dependent. Caterpillars injected with a smaller volume of extract (45µl) showed a smaller reduction in the amount of food eaten (0.7 times control), and the decrease was not statistically

significant. In this case, the time taken to reinitiate feeding did not differ from injected controls. Midgut injections of 90µl of the diet extract were more effective, delaying the reinitiation of feeding for a longer time (2.8 times saline control) than did equivalent injections of extract into the haemocoel. The 90µl injection into the midgut produced a similar decrease in meal size (0.3 times saline control) compared to the 90µl haemocoel injection. The impression that injections of diet extract into the midgut were more effective was confirmed by the effects of smaller injections. In contrast to the haemocoel injections , the injection of 45µl of the extract into the midgut was sufficient to produce a decrease in meal size of the same magnitude as the 90µl injection. Roughly half of the insects (40-50%) failed to feed within 30min of either the 45 or 90µl injections into the midgut.

It seemed possible that the effects of the diet extract injections might be a consequence of their ability to affect the osmotic pressure of either the gut or the haemolymph. Injections of 90µl of a xylose solution, with the same osmotic pressure as the diet extract, had no effect on the time interval before the start of feeding or the size of the subsequent meal. In this case, only 7% of the injected insects failed to feed. Therefore, the effects of the diet extract on feeding were due to the extract's nutrient content and not it's osmotic pressure.

Recurrent nerve operations

The foregut and midgut are innervated by the stomatogastric nervous system. The recurrent nerve is the principal route by which potential volumetric or nutrient feedback signals might be conveyed from the gut to the brain. The importance of such signals in the control of feeding behaviour was investigated by sectioning the recurrent nerve just behind the frontal ganglion and observing subsequent feeding behaviour.

The meal sizes of sham-operated (18 $\pm$ 4mg, mean $\pm$ SE, n=10) and recurrent nerve-cut insects (10 $\pm$ 2, mean $\pm$ SE, n=9) were not significantly different (t-test, $p>0.05$) when the operated insects were returned to their food after approximately 2h of starvation. This finding implies that neuronal activity in the recurrent nerve is unimportant in the control of normal feeding behaviour. However, it should be noted that the operations resulted in a

decrease in meal size of both sham-operated and recurrent nerve-cut insects compared to unoperated insects starved for a similar length of time (0.2 and 0.1 times unoperated controls, respectively). It thus remains possible that the physical trauma of the operations obscured the physiological function of the recurrent nerve in controlling food intake.

Discussion

Meal size and gut emptying in relation to volumetric feedbacks

Manduca caterpillars ate larger meals than usual when they were returned to their food after being starved for periods between the average meal interval (45min) and 120min. Longer deprivation did not result in any further increase in meal size. The increase in meal size with time since the last meal is consistent with the previously observed positive correlation found in normal feeding behaviour between interbout gaps of increasing length and the lengths of the following bouts (chapter 2, Reynolds *et al.*, 1986). A similar progressive increase in the amount eaten after longer intervals without food has been found in locusts (Louveaux, 1977) and also in various vertebrates (Levitsky and Collier, 1968).

Volumetric feedback from gut stretch receptors is well extablished as one source of information about the nutritional status of mammals which is used to control meal size (Davis *et al.*, 1975). Detailed work on locusts and blowflies has identified volumetric feedback from stretch receptors in the gut as being involved in the control of meal size (Gelperin,1971; Simpson, 1983). However, there are some important differences in gut structure and function between caterpillars and insects such as locusts and caterpillars (Chapman,1985; Dow,1986) which are likely to affect the usefulness of volumetric feedback as a factor in regulating food intake .

Briefly, the lepidopteran gut consists of a short, narrow foregut, a large midgut and a shorter hindgut. In caterpillars, the foregut is emptied rapidly following feeding and the rapid movement of food from the foregut to the midgut has been shown both here for *Manduca*, and also in another lepidopteran *Bombyx mori* (Hukahara *et al.*, 1981). In *Bombyx*, food was detected in the midgut within 1min of feeding.By contrast, the foregut of locusts and blowflies forms a capacious crop and is used to store and partially

digest food. Food may be held in the crop of locusts for 3h (Baines *et al.*, 1973; Roessingh and Simpson, 1984). The midgut is less well-developed in locusts and blowflies than in caterpillars. This is probably a consequence of the large amount of ingested food that needs to be broken-down and absorbed in order to maintain the high growth rates observed in caterpillars (Bernays, 1986).

Volumetric feedback from the foregut has been shown to exert a strong influence on the control of meal size in locusts and blowflies. An increase in the meal size of locusts with time since the last feed (Bernays and Chapman, 1972b) was suggested to compensate for the corresponding decrease in crop weight also observed. Volumetric feedback from stretch receptors in the locust crop has also been more directly implicated in the control of meal size: Bernays and Chapman (1973) found that cutting the posterior pharyngeal nerves which innervate the crop leads to an increase in food consumption. The amount of sugar solution drunk by the blowfly, *Phormia regina* , has been related to information from stretch sensitve neurones associated with the foregut (Dethier, 1967; Gelperin, 1971). In con trast to this rather clear picture, in *Manduca* the decrease in foregut weight following feeding showed no clear relationship to the observed increase in food intake after 45min or more without food. However, it would be unwise to ignore the possibility that volumetric feedback from the foregut may be involved in controlling feeding behaviour since it could be important in determining how much is eaten in a normal-sized meal.

Volumetric information concerning the bulk of material in the mid- and hindgut has also been shown to influence feeding in locusts. Roessingh and Simpson (1984) found that the midgut of *Schistocerca gregaria* nymphs was emptied of food by 8h after the last meal and this coincided with the biggest meal eaten by these insects. In addition, cannulations of agar and paraffin into the mid- and hindgut reduced the meal size of nymphs (*ibid*). The weight of the midgut of *Manduca* caterpillars decreased (by 27%) within a short period between 30 and 45min after the last meal, and thereafter remained constant. Meal size increased between 45 and 120min after feeding and although this did not coincide exactly with the decrease in midgut weight observed in this experiment, the disparity was not great. Additionally, it may be significant that the increase in meal size was similar (55mg) to the decrease in midgut weight (79mg). Hence, it is possible that volumetric feedback from the

midgut may have a function in controlling meal size in caterpillars.

The separate portions of the gut take several hours to empty in locusts (Baines *et al.*, 1973) but the rate of gut emptying following feeding in *Manduca* caterpillars was more rapid. If negative feedback from gut stretch receptors was important in determining the amount eaten in a meal, then the rate at which this inhibition was relaxed might affect the initiation of feeding. Such a mechanism would be possible in *Manduca* since decreases in the weight of fore- and midgut were noticable at approximately the same time as the mean intermeal interval for day 0 insects. Signals from receptors monitoring nutrient levels in the blood may also be important in controlling feeding behaviour. Such signals may interact peripherally with volumetric feedback. Changes in blood composition after feeding have been suggested to influence the rate of gut emptying in insects (see Bernays and Simpson, 1982), although recent studies have not supported such an effect in locusts (Abisgold and Simpson, 1987).

Where nutrient and volumetric feedbacks have independent access to the decision-making centres that control feeding, their effects might be combined and some evidence for a set point controlling meal size in *Manduca* larvae was discussed in chapter 2. Hence, both nutrient and volumetric feedbacks may influence the food intake of *Manduca* caterpillars.

Control of food intake by nutrient and volumetric feedbacks: evidence from injection experiments

Two firm conclusions can be drawn from the results of the experiments designed to alter the volume and/or nutrient content of the gut and haemolymph of *Manduca* caterpillars. First, nutrient feedbacks from the haemolymph and midgut are involved in the control of meal size and the time between meals. Secondly, the responses to increased nutrient levels are specific nutrient effects and are not due to changes in osmotic pressure.A less certain outcome of these experiments relates to the role played by volumetric feedback. Injections of inert material into the gut failed to produce any evidence that volumetric feedback limits meal size. However, this possibility cannot be completely excluded.

Data relating to changes in the levels of haemolymph nutrients following feeding in *Manduca* larvae have already been presented and discussed (see chapter 5). Variations were observed in the levels of carbohydrates and free amino acids during and after the meal. Attempts were made to test for an effect of blood glucose and amino acid concentrations on the time between meals, but no significant effects were observed. The limitations of those experiments were discussed in chapter 4. Abisgold and Simpson (1987) showed that raising the amino acid concentration in the haemolymph of locusts prevented 79-80% of insects from feeding within 30-40min of being injected. In the present study with *Manduca*, the time taken to start feeding was significantly delayed after the injection of diet extract, but only by a matter of seconds and certianly not by a margin approaching the normal intermeal interval of *Manduca* caterpillars. The physiological relevance of such an effect is, therefore, more difficult to interpret.

Much more importantly, injections of the diet extract into the haemolymph also caused a marked reduction in meal size. No effect of osmotic pressure alone on meal size was observed so that the effect of the diet extract must have been due to it s content of nutrients, and not simply due to it's osmotic effects. This result contrasts with studies on locusts where increased blood osmolality has been shown to reduce meal size (Bernays and Chapman, 1974a; Roessingh *et al.*, 1985). Altering the nutrient content of the midgut also affected the likelihood of feeding and the amount eaten. The satiating effects on feeding of nutrients introduced directly into the gut have been studied extensively in vertebrates. Particular attention has been paid to glucose infusions into the duodenum and hepatic portal blood system (Campbell and Davis, 1974; Novin *et al.*, 1974).Basically, the results suggest that gastric and hepatic glucosereceptors may be involved in controlling food intake in vertebrates.

Levels of haemolymph and midgut nutrients might influence the initiation and termination of feeding in caterpillars by several possible mechanisms. First, they may act directly upon the CNS. An effect of this kind has been observed in the slug, *Limax maximus* (Phifer and Prior, 1985). In this case, raising the blood osmolality reduced neuronal activity in the buccal ganglion and consequently the feeding responsiveness of the slug. Second, the CNS could be stimulated indirectly as a result of peripheral effects. Such a mechanism has recently been described in locusts. Simpson and Abisgold

(1988) showed that the sensitivity of gustatory sensilla on the mouthparts responds to amino acid levels in the blood, thus, altering the nature of taste information relayed to the CNS. Alternatively, both these mechanisms could be involved. It is in any case very difficult to know whether the effects of haemolymph and midgut injections in *Manduca* were the results of similar physiological processing. For example, it is possible that the effects produced by injections into the midgut were actually the results of changes in the composition of the haemolymph that ensued after the extract was digested and absorbed.

The morphology of the cerebral neuroendocrine system in *Manduca* larvae has been described (Copenhaver and Truman, 1986) but details of the stomatogastric system are more limited. However, some unpublished observations (pers. comm. P.H. Taghert, Washington University, St. Louis, Missouri, U.S.A.) suggest several interesting features associated with the gut. Numerous neuronal cell bodies are apparently positioned along each of the 6 longitudinal muscle bands surrounding the midgut. These have axons projecting to the CNS, either forward *via* the frontal ganglion (front 2/3rds of the midgut) or backward *via* the terminal abdominal ganglion (rear 1/3rd of midgut). Motorneurones in the frontal ganglion innervate both the muscles in the foregut and the sphincter separating the fore- and midguts. Finally, there also appear to be cells in the midgut epithelium resembling endocrine cells in their staining properties and ultrastructure. Although some of these gut endocrine cells stain with specific antisera (*e.g.* raised against a molluscan myotropic peptide), it is not known whether this peptide is actually present in these cells.

Hence, neurones on the surface of the midgut could respond to physical changes in the volume of the midgut and/or monitor changes in the chemical composition of the haemolymph. Information concerning such internal variables could be relayed directly back to the CNS *via* axons connected to the recurrent nerve. In insects there are no reports of internal taste receptors but intestinal glucoreceptors (Mei, 1978) and amino acid receptors (Jeanningros, 1982) have been identified in cats. Additionally, endocrine cells within the gut wall might be able to detect subtle changes in the nutrient status of the midgut contents and pass hormonal messages back to the CNS. The presence of endocrine-like cells of unknown function has been documented in

the midgut of the cockroach, *Periplaneta americana* (Endo and Nishiitsutsuji-Uwo, 1982). In vertebrates, it is believed that food in the intestine stimulates the release of a hormone that induces satiety (Gibbs *et al.*, 1973; Liebling *et al.*, 1975).

Possible routes for the passage of nervous signals or even neurosecretory material (Khan, 1976) to and from the CNS are likely to involve the recurrent nerve. If information concerning possible negative feedback was communicated *via* this pathway in *Manduca* caterpillars, then severing the recurrent nerve ought to lead to increased food intake. The fact that there was no difference in meal size between sham-operated and recurrent nerve-cut insects suggests that either this mechanism does not operate in *Manduca* or that the response was overridden by other influences. Such factors might include the physical trauma of the experiment and the lack of sufficient time allowed for recovery after the operations. Another possibility is that the normal feeding response was impaired because salivation and/or foregut emptying were disrupted. Casual observations of caterpillars with severed recurrent nerves support this suggestion. After several days of feeding on normal diet the insects' food appear ed to be covered in regurgitated food. Although more extensive studies are required to confirm this observation, it seems likely that severing this nerve might have interupted the swallowing of the food and interfered with salivation. This is certainly possible in *Manduca* since the salivary nerves are innervated solely by the recurrent nerve (Robertson, 1974).

Other physiological mechanisms may also be involved in the control of feeding in caterpillars. For example, feedback involving total body volume (Moorhouse *et al.*, 1976) and sensory adaptation of mouthpart taste receptors (Barton Browne *et al.*, 1975b) have been connected with the regulation of meal size in locusts. An important possibility overlooked in the present study was the involvement of the hindgut in determining how much and how often caterpillars eat. Volumetric feedback from the hindgut has been shown to influence meal size in *L. migratoria* nymphs (Simpson, 1983). Simpson and Ludlow (1986) also demonstrated that defaecation increased the probability of feeding by seven-fold. Reinecke *et al.* (1973) described a sensory neurone associated with the proctodael nerve in fifth instar *Manduca* larvae and this has been suggested to function as a stretch receptor controlling the formation

of faecal pellets. Perhaps, this system is analogous to the stretch receptors which have been identified on the posterior ileum in locusts (Simpson, 1983), and which monitor the fullness of the hindgut and affect meal size. Hence, although a variety of other factors may be involved, nutrient feedbacks appear to play a significant role in the control of feeding in *Manduca* caterpillars and the nature of this effect necessitates further study.

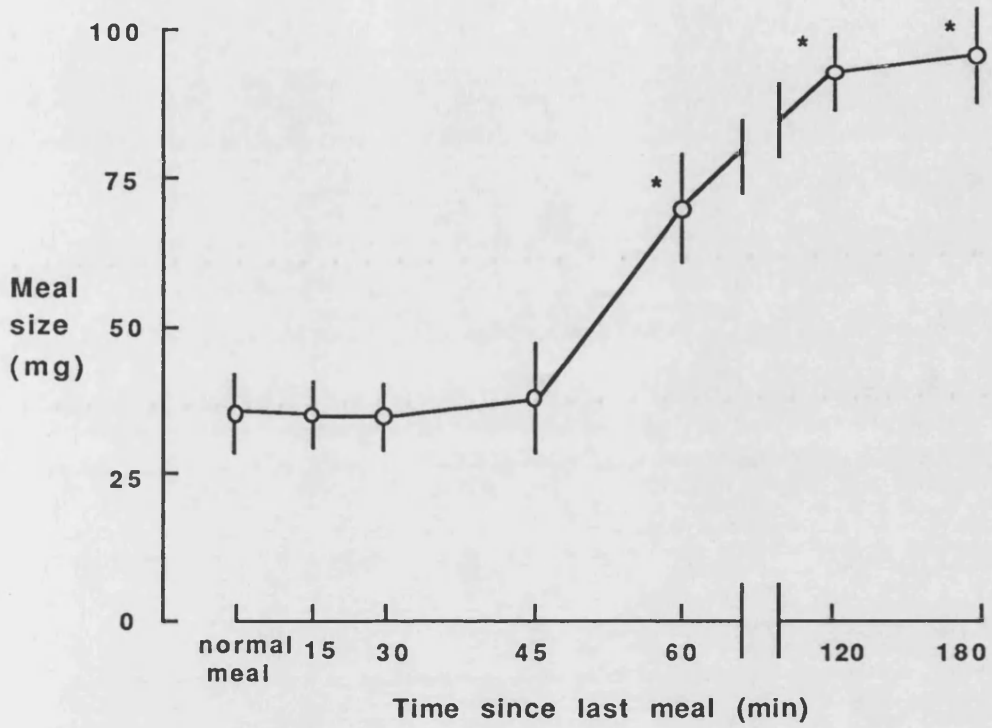


FIG. 6.1. The size of the first meal taken by day 0 fifth stadium Manduca sexta larvae given artificial diet after different periods of food deprivation. The caterpillars were removed from the diet 10min after the end of the previous meal (10min is the bout criterion) and then held without food until the time shown on the abscissa. Thus, the period during which the caterpillars did not feed is that shown, although the period during which they were denied access to food is 10min less than that shown. Means ±SE (n=10 per point).

* Denotes a significant difference (one-way ANOVA, 95% confidence intervals based on pooled SD) between the indicated point and the value at the end of a normal meal (i.e. the caterpillar was kept in contact with the food after the end of the meal until the bout criterion had elapsed).

FIG.6.2. Changes in (a) fore-, (b) mid- and hindgut weights in day 0 fifth stadium *Manduca sexta* larvae given normal diet after different periods of food deprivation. The caterpillars were removed from the diet 10min after the end of the previous meal (10min is the bout criterion) and then held without food until the time shown on the abscissa. Thus the period during which the caterpillars did not feed is that shown, although the period during which they were denied access to food is 10min less than that shown. Means ± SE (n=10 per point).
* Denotes a significant difference (one-way ANOVA, 95% confidence intervals based on pooled SD) between the indicated point and the value at the end of the meal ('E+10'). 'S' represents the start of the meal.

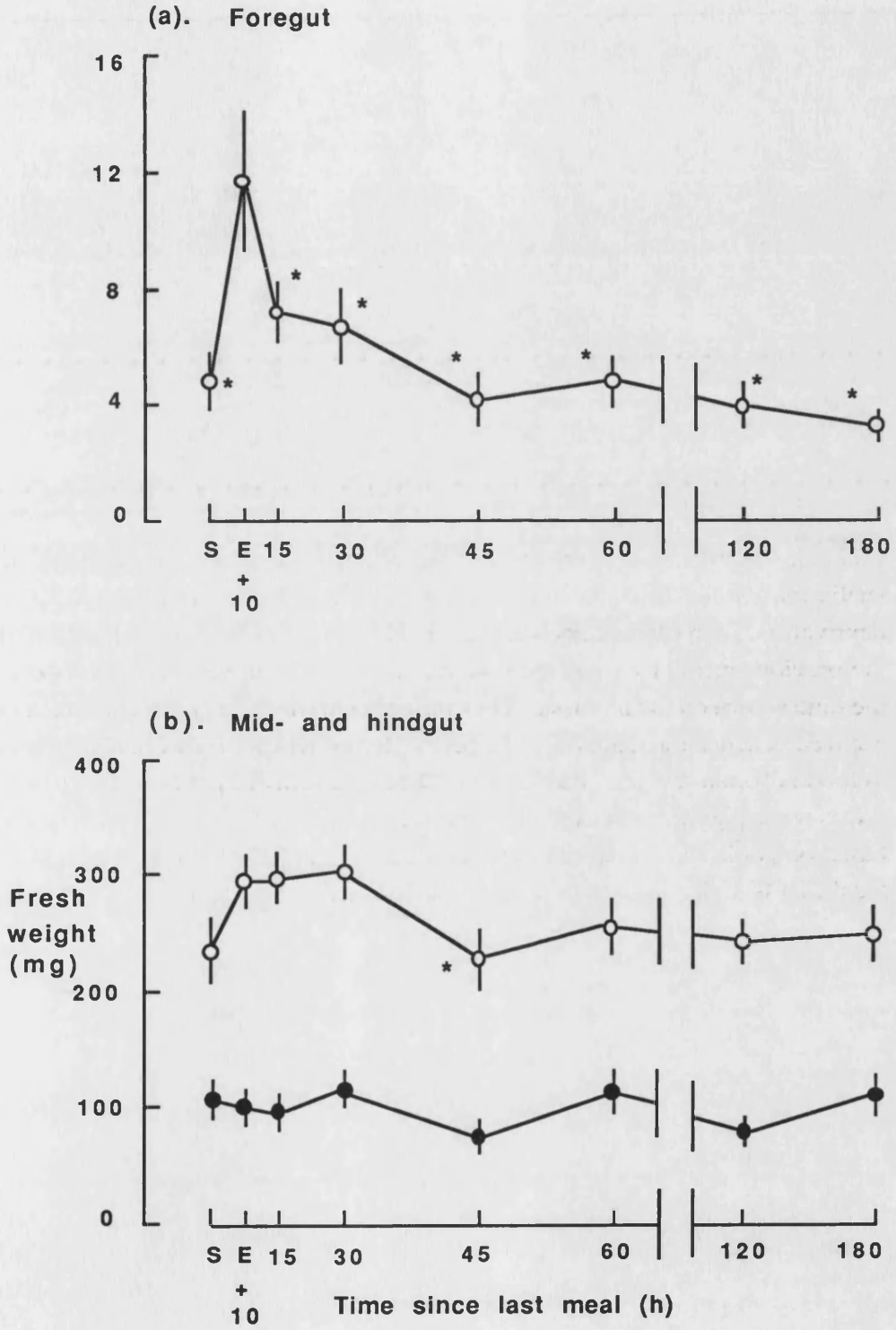

(a). Foregut
16
12
8
4
0
S
E
+
10
15
30
45
60
120
180
(b). Mid- and hindgut
400
300
200
100
0
Fresh
weight
(mg)
Time since last meal (h)

FIG.6.3. The effects of injecting different volumes of a paraffin mixture into the fore- and midgut on the feeding parameters of day 0 fifth stadium *Manduca* larvae. (a) % failing to feed within 30min of injection. (b) time to start feeding after being placed back on the diet after injection (c) meal size after injection. The height of each bar represents the mean ± SE. 10-15 insects were used for each injection and the number which fed are given in the open bars. For each region of the gut, significant differences between injections are indicated by different superscripts (one-way ANOVA, 95% confidence intervals based on pooled SD).

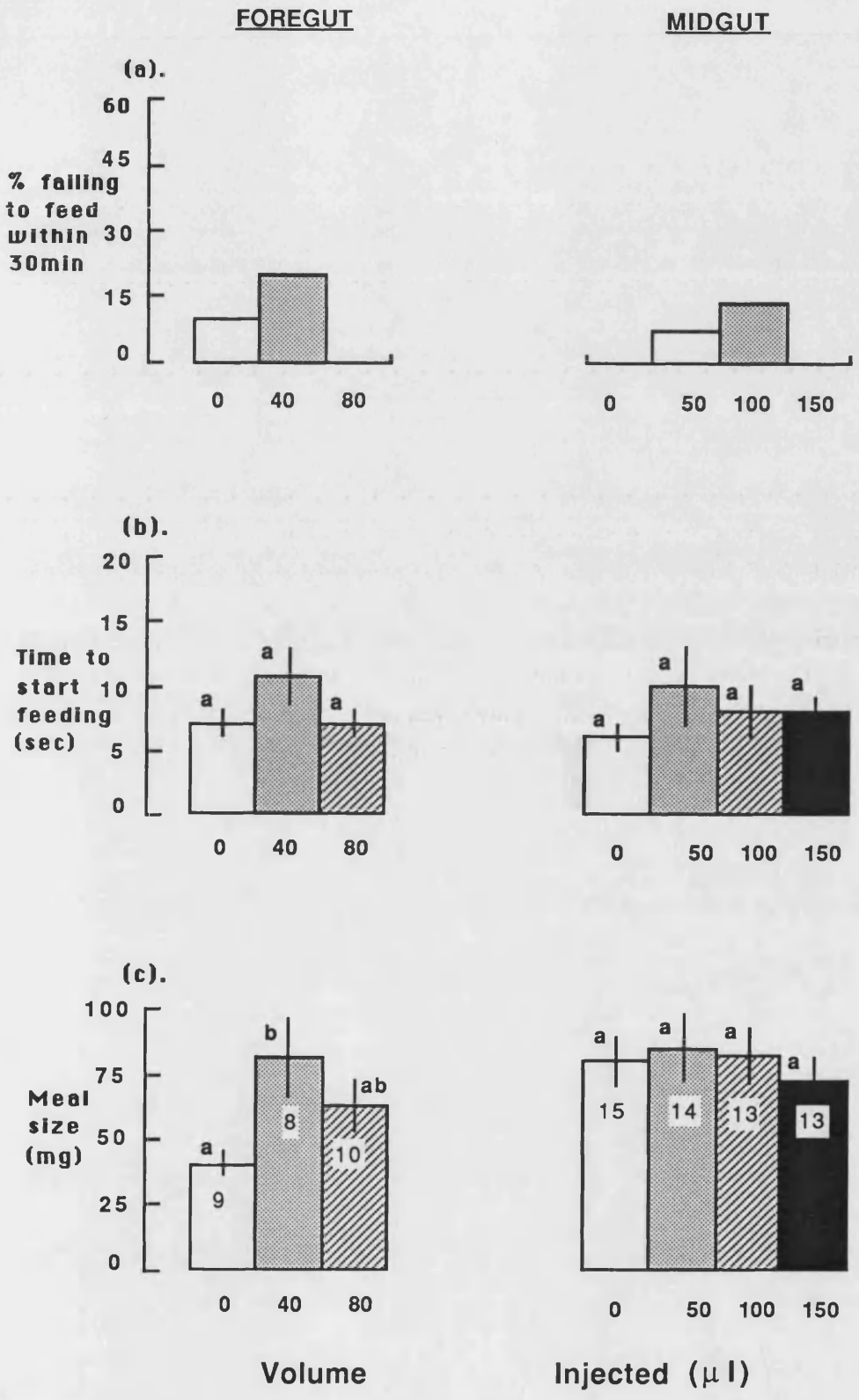

FOREGUT
MIDGUT
(a).
60
45
% failing to feed within 30min
30
15
0
0 40 80
0 50 100 150
(b).
20
15
Time to start feeding (sec)
10
5
0
a a a
a a a a
0 40 80
0 50 100 150
(c).
100
75
Meal size (mg)
50
25
0
a b ab
9 8 10
a a a a
15 14 13 13
0 40 80
0 50 100 150
Volume
Injected (µl)

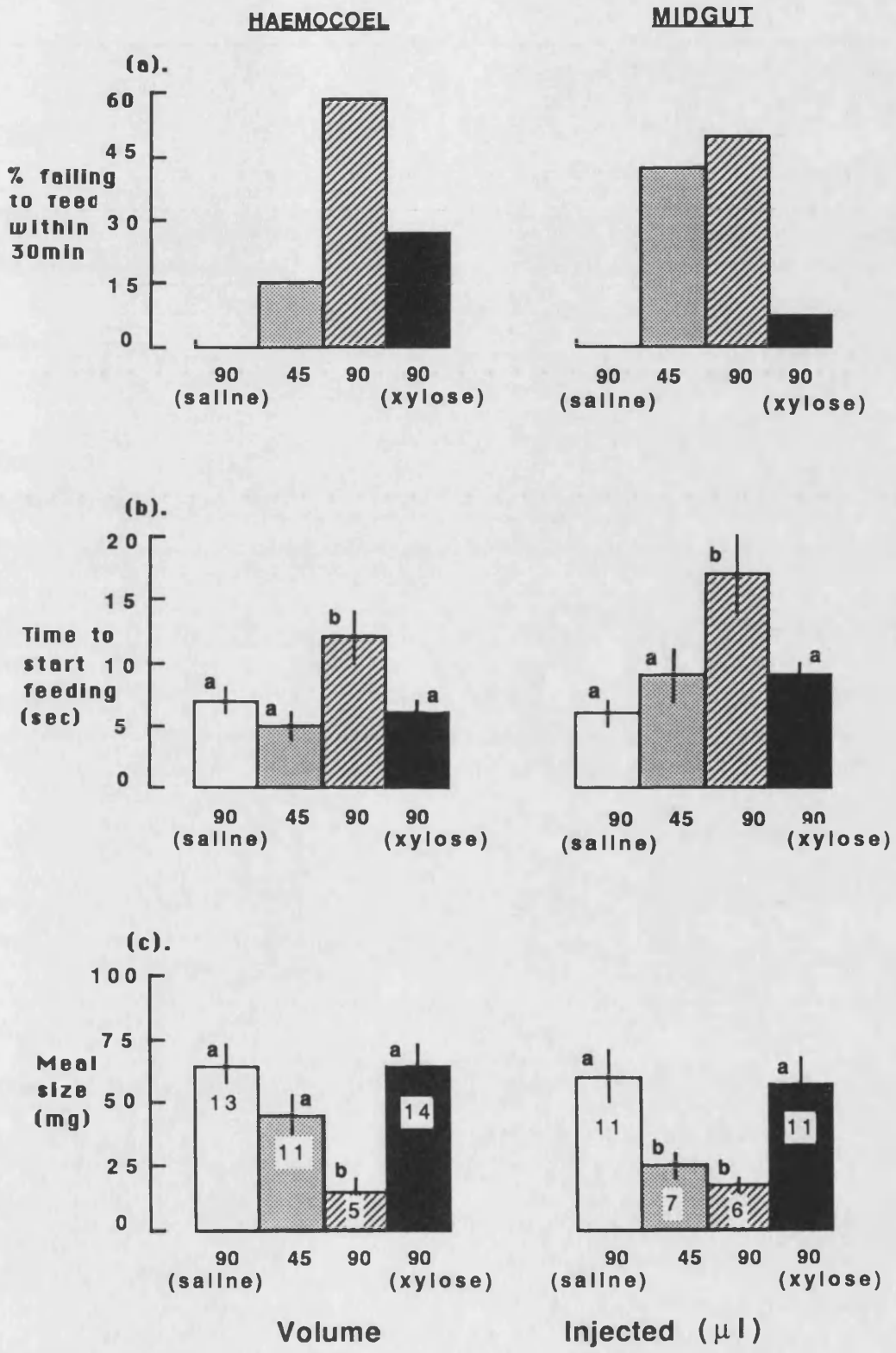
FIG.6.4. The effects of injecting different volumes of a crude diet extract into the haemocoel and midgut on the feeding parameters of day 0 fifth stadium *Manduca* larvae. (a). % failing to feed within 30 min of injection. (b). time to start feeding after being placed back on the diet after injection. (c). meal size after injection. The height of each bar represents the mean ± SE. 10-15 insects were used for each injection and the number which fed are given in the open boxes. For each region of the gut, significant differences between injections are indicated by different superscripts (one-way ANOVA,95% confidence intervals based on pooled SD).


saline control (90µl)


diet extract (45µl)


diet extract (90µl)


osmotic control (90µl xylose)


HAEMOCOEL
MIDGUT
(a).
% failing to feed within 30min
60
45
30
15
0
90 45 90 90
(saline) (xylose)
(b).
Time to start feeding (sec)
20
15
10
5
0
a
a
b
a
(c).
Meal size (mg)
100
75
50
25
0
13
11
5
14
11
7
6
Volume Injected (µl)

Chapter 7

Physiological effects of azadirachtin on growth in *Manduca sexta* larvae

Introduction

Azadirachtin is a complex terpenoid present in the fruits and other tissues of the tropical Neem tree (*Azadirachta indica*). It has been recognised to have formidable antifeedant and growth reducing properties against many species of insects (Kubo and Nakanishi, 1979; Warthen, 1979). Azadirachtin also represents the active ingredient in the Neem extracts which have been the subject of much interest as potential crop protection agents (Bernays, 1983).They would have advantages of low cost and lack of toxicity to vertebrates and could easily be produced by farmers in underdeveloped tropical countries (Schmutterer and Ascher, 1984).

The deterrent action of azadirachtin on feeding in insects has been shown to be due to both the stimulation of specific deterrent receptors and the inhibition of specific receptors which signal phagostimulants in the food (Schoonhoven and Jermy, 1977; Simmonds and Blaney, 1984). Azadirachtin appears to have several other properties apart from its antifeedant effects. In Lepidoptera, azadirachtin has been shown to interrupt the process of moulting in larvae of *Bombyx mori* (Koul *et al.*, 1987) and *Manduca sexta* (Haasler, 1984; Reynolds S.E. and Wing K.D., pers. comm.). The reproductive potential of insects can also be adversely affected by treatment with azadirachtin and Schlüter and Schulz (1984) showed that azadirachtin interfered with oogenesis and spermatogenesis in adult Mexican bean beetles, *Epilachna varivestis*.

Since azadirachtin is a potent feeding deterrent, it is important to

distinguish between the primary effects on growth produced after ingestion and those resulting from reduced food intake. There is some evidence that azadirachtin can impair the growth of insects without reducing food intake. For example, topical applications of azadirachtin have been shown to disrupt growth in *Ephestia kuehniella* (Sharma *et al.*, 1980; Rembold *et al.*, 1982). In this chapter, the effect of azadirachtin on the growth of *Manduca* caterpillars is investigated without direct feeding inhibition. The physiological basis of the effect on growth is examined.

Materials and methods

Growth, food intake and feeding behaviour in control and azadirachtin-treated insects

Insects were maintained until the day of ecdysis to the fifth-instar under standard conditions (see appendix 2). Newly moulted larvae, weighing 1.6-1.8g, were selected for injection within 2-3h of lights on (07:00h). Insects were injected with 5,10 or 25µg azadirachtin as a $1mgml^{-1}$ solution in 10% ethanol. Injection was between abdominal segments 4 and 5 using a 25µl Hamilton syringe. Control insects received 25µl of 10% ethanol solution. Nutritional indices were measured using the gravimetric method described in chapter 3. The absorption efficiency of nitrogen was measured on day 1 of the instar (*i.e.* approximately 24h after injection) as in chapter 4. The retention time of food in the gut and feeding behaviour of day 1 insects were recorded using the techniques described in chapter 3.

Measurement of respiration rates in control and azadirachtin-treated insects

The respiration rate of day 1 larvae, injected approximately 24h earlier with 0 or 25µg of azadirachtin, was measured using a Gilson differential respirometer (series G). Larvae were weighed to the nearest 1mg and placed in 100ml Warburg flasks containing a small test-tube of 20% KOH with a paper wick which absorbed any CO_2 gas. A small piece of diet weighing about 5mg was also placed in the flask was allowed 1h to equilibrate at 25 ±

2°C before any measurements were taken. The oxygen consumption of each animal was measured from the volume decreases occurring during successive 10min intervals and was expressed as the volume (ml) of oxygen consumed per g of larval tissue per hour (ml O_2 $g^{-1}h^{-1}$).

Measurement of the loss of radio-labelled glycine from the whole gut of control and azadirachtin-treated insects with time since the last meal

Artificial diet was prepared according to the procedure described in appendix 1, except that the premix (336g) and distilled water (700ml) were cooked for 10min in a microwave to kill yeast cells in the premix that might digest the labelled amino acid. A sufficient quantity of [^{14}C] -glycine (Amersham) was added to the premix/water mixture to produce an approximate concentration of 0.1μCi per ml of liquid diet. The label was mixed thoroughly with the diet for 10min before the other ingredients were added.

Insects which had been injected 24h previously with 0 or 25μg of azadirachtin were observed feeding on normal diet until they ate a meal of at least 4min followed by 6min (approximate bout criterion for control and treated insects- see Table 7.2). They were then removed and left for 45min without food (approximate gap length for control and treated insects- see Table 7.2). Caterpillars were weighed to the nearest 1mg and allowed to complete a meal on the labelled diet followed by 6min without feeding. They were then reweighed so that the meal size (and therefore the amount of [^{14}C]-glycine eaten) could be calculated. Insects were deprived of food for various periods up to 24h.

The whole gut was quickly removed by anaesthetising with CO_2 gas (30 sec in a beaker with a small quantity of dry-ice) and divided into sections: 1. foregut (oesophagus and pharynx); 2. anterior third of the midgut; 3. middle third of midgut; 4. posterior third of midgut; 5. and hind gut (ileum, rectum, colon). 2.5ml of phenylthiourea was added to each gut section (to prevent blackening of the samples due to tyrosinase in the haemolymph) and the sections were homogenised by grinding with a glass rod for 5min. The suspension was centrifuged (Beckman J-6 centrifuge) at 3000 times g(force of gravity) for 15min and the supernatant removed. This extraction procedure

was repeated a second time and the supernatants combined and dried-down overnight under reduced pressure (Speedvac, Savant). The residue was resuspended in 1.0ml of distilled water and 500µl of the suspension was added to 5.0ml of scintillant (Optiphase 'safe', LKB). The samples were counted on a LKB Rackbeta-1217 liquid scintillation counter which used a quench correction curve previously constructed from a range of gut sample dilutions. Samples of the labelled diet were taken through the same procedure so that the amount of label eaten could be calculated.

Measurement of the midgut trypsin-like activity of control and azadirachtin-treated insects

Insects which had been injected 24h previously with 0 or 25µg of azadirachtin were sacrificed and the midgut removed. The midgut was cut open and the contents placed in an eppendorf tube (1.5ml) which was centrifuged for 1min at 3000 times g(force of gravity). A 10µl aliquot of the supernatant was used in the enzyme assay. The gut wall was washed in insect saline (Ephrussi and Beadle, 1936) and homogenised (homogensing tube) with 1.0ml of buffer (2mM $CaCl_2$, 100mM Tris, pH 9.0). The suspension was centrifuged for 5 min at 1300 times g(force of gravity) and a 100µl aliquot used in the enzyme assay. For the assay, modified from Santos *et al.*, 1983), 990µl of buffer and 250µl of the substrate (30mM BAPNA in dimethyl formamide) were added to 10µl or 100µl of the sample in a cuvette. The cuvette was shaken and immediately transferred to a spectrophotometer (Cecil CE373) which was connected to a pen recorder. The rate of change of optical density was measured at 410nm and was assessed in terms of the slope of the traces from the pen recorder. The trypsin activity was expressed as the BAPNase activity (nmol $min^{-1}ml^{-1}$) of the midgut wall. The effect of nutritional status on the trypsin activity of the midgut and it's contents was tested by starving insects for 24h immediately after being injected and repeating the above assay.

An attempt to measure trypsinogen-like activity in the gut wall was made by incubating 500µl of the gut wall extract from untreated insects in 1.0ml of $CaCl_2$/Tris buffer for 24h at 4°C and repeating the trypsin assay (Willimowska-Pelc and Mejbaum-Katzenellenbogen, 1978).

The midgut pH of control and azadirachtin-treated insects was measured on day 1 of the fifth stadium, approximately 24h after the injection of 0 and 25µg of azadirachtin. Excess haemolymph was removed by blotting the midgut on tissue paper and the midgut contents were then tansferred to an eppendorf tube. The pH was read using a digital pH meter equipped with a semi-micro combination pH electrode (Russell).

Midgut histology of control and azadirachtin-treated insects

The histology of the midgut epithelium of day 1 larvae (injected 24h previously with 0 and 25µg of azadirachtin) was examined. The midgut was removed and 1mm wide rings of midgut tissue cut from the anterior, middle and posterior thirds of the midgut. The rings of tissue were washed in saline (Ephrussi and Beadle, 1936) in order to remove the gut contents, and were then fixed overnight at 4°C in 4% glutaraldehyde buffered with 0.1M sodium cacodylate at pH 7.4. The tissue was then postfixed in two changes (1h each) of 1% osmium tetroxide in the same buffer. After a brief rinse with buffer, the tissue was dehydrated through a graded acetone series: 30%, 50%, 70% (2 rinses of 15min at each dilution) and 100% (2 rinses of 1h each).

The preparations were embedded overnight at room temperature in a 1:1 (v/v) mixture of 100% acetone and resin (TAAB). The tissue was taken through 2 changes of 100% resin and finally cured with fresh resin at 60% for 48h. For light microscopy, 1µm thick sections were cut using a Reichert (0mu3) ultramicrotome and the sections were stained with 1% toluidine blue in a 1% borax solution. Photomicrographs were taken using a Carl Zeiss photomicroscope. 1nm thin sections were cut for electron microscopy and double stained with uranyl acetate and lead citrate (Reynolds, 1963). Electron micrographs were taken using a Jeol (100CX) electron microscope.

Results

Effects of azadirachtin on growth and food intake

When fifth stadium *Manduca* caterpillars were injected with increasing

doses of azadirachtin, a dose-dependent reduction in the rate of fresh weight gain and a consequent lengthening of the feeding period were observed (Fig.7.1). At doses of 10 and 25μg of azadirachtin per larva, the growth rates (in dry weight terms) were significantly decreased, repectively, to 0.6 and 0.5 times the control value, respectively. The effect at a dose of 5μg was not significant, however, in dry weight terms.

The ability of azadirachtin to inhibit growth was not a consequence of reduced food intake as the consumption rates (dry matter) of caterpillars were unaffected by treatment with azadirachtin at any of the doses used in this study (Table 7.1). The lack of an effect on the amount of food eaten was supported by an examination of the amount of time spent feeding on day 1 of the fifth stadium (Table 7.2). Control and azadirachtin-treated (25μg) caterpillars spent the same amount of time feeding. Although the gap lengths of treated insects were significantly increased (1.3 times control), bout length was unaffected and bout frequency remained unchanged.

Efficiency of food use and respiration rates

At levels of azadirachtin which adversely affected growth, the overall efficiency of conversion of food into biomass (ECI) was significantly reduced (Table 7.1). This was a consequence of a reduction in the efficiency of nutrient utilisation (ECD) which was significantly decreased to 60-70% of the control value. Reduced values of ECD must result from a greater proportion of the absorbed nutrients being used for purposes other than growth. These might include energetically costly metabolic processes (such as gluconeogenesis), increased turnover of body components (this might include the synthesis of digestive enzymes), and increased expenditure of energy in muscular activity (whether this is related to the acquisition of food or not). Although azadirachtin did not affect the amount of food consumed by *Manduca* caterpillars, energy expended on, for example, increased locomotor activity would reduce ECD. However, behavioural activities other than feeding were not quantified. In this case, an additional possibility is that energy might be required to detoxify azadirachtin in treated insects.

Oxygen consumption in azadirachtin-treated (25μg) insects (1.05 ± 0.05 $mlO_2g^{-1}h^{-1}$, mean ± SE, n=10) was slightly higher (1.2 times) than in controls

(0.91 ± 0.13 $mlO_2g^{-1}h^{-1}$, mean ± SE, n=10) although the difference was not statistically significant (t-test, $p>0.05$). This would represent a decrease in ECD from 47% to 38%, rather than the 29% actually observed in the 25µg-treated insects (Table 7.1). The effect of this small difference might be compounded as insects progress futher through the instar, since the 'energetic costs' of growth have been shown to increase (Reynolds *et al.*, 1985). Additionally, since azadirachtin prolongs the instar, the increased costs will also be incurred over a longer period.

In addition to changes in the efficiency at which food is converted into growth, reductions in the efficiency at which nutrients are extracted from food in the gut could also impair growth. Estimates of the approximate digestibility of dry matter, AD (dry matter) were unaffected by treatment with azadirachtin. Hence, treated insects apparently absorbed dry matter (nutrients) at the same efficiency as control insects. But, it remains possible that the uptake of individual nutrients was affected.

A nutrient that is known to limit the growth of *Manduca* caterpillars is nitrogen (see chapter 4). The faeces of day 1 insects, injected 24h previously with 25µg azadirachtin, contained 78% more nitrogen (% dry weight nitrogen content 2.26 ± 0.16, mean ± SE, n=5) than those of controls (% dry weight nitrogen content 4.02 ± 0.17, mean ± SE, n=5). The increased faecal nitrogen content was not due to a greater production of uric acid, since the levels of uric acid were low in the faeces of both groups (about 0.1% dry weight). Hence, the efficiency at which nitrogen was abosorbed from the food, AD (nitrogen), was significantly lowered (t-test, $p<0.05$) in azadirachtin-treated insects (50.69 ± 8.46%, mean± SE, n=10) compared with control insects (71.29 ± 4.71%, mean ± SE, n=10). Even though the value of AD (nitrogen) for treated caterpillars was reduced to 0.7 times the control value, no significant decrease in the estimate of AD (dry matter) was observed. This discrepancy could possibly have arisen if treated insects increased the amount absorbed of other nutrients from the food, such as carbohydrates and lipids. Unfortunately, absorption efficiencies for these nutrients were not measured. Alternatively, a change in the efficiency of handling nitrogenous nutrients alone might have been insufficient to register a significant change in AD (dry matter). Using a value of 30% protein content (dry weight) for normal artificial diet (see chapter 4) it can be calculated that this decrease in AD (nitrogen) would cause a reduction of 5% in AD (dry matter). Infact, this value lies within the limits

of statistical variation associated with the mean value of AD (dry matter).

Retention times

Table 7.1 shows the retention times of food in the gut of control and azadirachtin-treated insects. When caterpillars were injected with a low dose of azadirachtin (5μg per larva), the speed of passage of the food through the gut was increased to 1.7 times the normal value. However, at higher doses this effect was not maintained and food remained in the gut for the same average length of time as control insects.

Histology of midgut epithelial cells

As in most lepidopteran larvae, the midgut of *Manduca* caterpillars is the main site of digestive enzyme secretion and nutrient absorption (Turunen, 1985). There are two principal types of cell in the midgut of *Manduca* : the goblet cells (thought to regulate the fluid and ionic environment of the midgut, Gupta *et al.*, 1985); and the more numerous supporting columnar cells (Cioffi, 1979). The structural arrangement of these cell types in 3 regions (anterior, middle and posterior) of the midgut epithelium is shown for control and azadirachtin-treated insects in Figs.7.2-7.4.

In controls, the apical (lumen) surface of the midgut epithelium is bordered in all 3 regions by regular brush border of microvilli. This decoration of microvilli is associated with the columnar cells (Figs.7.2a,7.3a,7.4a). Associated with this border were apical blebs and secretory vesicles representing the presumed result of holocrine secretion (Wigglesworth, 1965). The distribution of this presumed secretory activity throughout the midgut agrees with the similar situation found in a related sphingid moth, *Erinnyis ello* (Santos *et al.*, 1984).

In contrast, the brush border of treated insects was irregular and most of the columnar cells showed gross swellings of the apical region (Figs.7.2b,7.3b,7.4b). The goblet cells, on the other hand, did not show such conspicuous morphological alterations. This effect was consistently observed in sections from five different control and treated insects. Although no attempt was made to quantify the relative extent of the 'swollen blebbing' that

observed in treated insects or the secretory activity seen in control inects, it is clear the normal blebbing and release of secretory vesicles are replaced in azadirachtin-treated insects by gross apical blebbing.

The examination of columnar cells (middle region of the midgut) at higher magnifications using elecron microscopy revealed more differences in ultr-structure between control and treated insects (Figs.7.5-7.7). In general, the cytoplasmic material was more densely-packed in the apical area of columnar cells of control insects. Areas devoid of definite cell structures were evident in treated insects (Figs.7.5b,7.6b,7.7b). Filamentous threads of rough endoplasmic reticulum were apparent together with many secretory vesicles. In contrast to the controls, mitochondria were less numerous in the apical cytoplasm of azadirachtin-treated insects.

Trypsin-like enzyme activity

The above differences in midgut ultrastructure implied that inhibition of normal enzyme secretion may have occurred as a result of treatment with azadirachtin. Additionally, caterpillars treated with azadirachtin experienced reduced efficiencies of nitrogen absorption (see above). Trypsin-like enzyme activity has been shown to be high in the midgut contents and wall of a related Sphingid moth, *Erinnyis ello* (Santos *et al.*, 1983). Hence, the activity of trypsin-like enzymes in the midgut of control and treated inects was compared.

A much reduced level (0.2 times control) of trypsin-like activity was found in the midgut contents of treated caterpillars (Fig.7.8a). The reduction in the trypsin-like activity of the midgut contents was not accompanied by any significant change in the amount of enzyme present in the midgut wall (Fig.7.8b). To ensure that this difference was not a consequence of the impaired nutritional state of the treated caterpillars, untreated insects were starved for 24h and their enzyme-activities compared with fed-control insects. This did not affect the trypsin-like activities of the midgut contents or wall (Fig.7.8a and b). The reduction in enzyme activity in the midgut contents of the treated insects was not due to direct inhibition of trypsin by azadirachtin. When a high concentration of azadirachtin of roughly 2 times the maximum dose injected (*i.e.* 50µg per larva) was added to the midgut contents, BAPNase activity remained unaltered.

The reduction in trypsin activity in the midgut contents of azadirachtin-treated insects might have resulted from a failure to secrete trypsinogen, which would thus have accumulated in the midgut wall. To test this, an attempt was made to assay trypsinogen by incubating the gut wall extracts in the $CaCl_2$/Tris buffer overnight at 4°C. Such treatment did not lead to a significant increase in BAPNase activity in the extracts, and it is tentatively concluded that there is no evidence for the presence of trypsinogen in the *Manduca* midgut wall.

Trypsin-like activity in the midgut contents was measured under standard conditions, at pH9.0, close to the enzyme's optimum. *In vivo*, however, pH might have varied in the azadirachtin-treated insects. When checked, the pH of the gut contents was found to be the same for both groups of insects (control pH 9.03± 0.09, mean ± SE, n=10; 25µg azadirachtin pH 9.00 ± 0.15, mean ± SE, n=10) so that the usual pH of enzyme activity in the gut was maintained.

Absorption of [^{14}C]-glycine

As well as suggesting a negative effect on digestive enzyme secretion in the gut, changes in the detailed structue of the midgut epithelium could also have affected nutrient absorption. This was checked by measuring the time course of removal of [^{14}C]-glycine from the guts of control insects and insects injected with 25µg of azadirachtin. The results are shown in Fig.7.9. Within 30 min of the end of feeding, 40% of the label had been absorbed from the guts of both groups of insects. A more gradual uptake of the label from the gut followed this initial decrease, and about 80% was removed from both treated and untreated insects by 24h after the labelled meal. Thus, treatment with azadirachtin, at sufficient dose to reduce normal growth, did not affect either the rate of absorption of [14C]-glycine from the gut or the final percentage of the labelled-meal remaining in the gut at 24h following feeding.

Discussion

Growth and the efficiency of food utilisation and uptake

Azadirachtin impaired the growth of *Manduca* caterpillars and this effect was not due to a reduced intake of nutrients in the food. Azadirachtin has been shown to inhibit growth without an antifeedant effect in insects belonging to several different orders (Sharma *et al.*, 1980; Rembold *et al.*, 1982). In such cases, larval weight gain was measured following topical applications of azadirachtin but no estimate was made of food intake or food use. The only detailed studies which have attempted to examine the nutritional effects of azadirachtin have involved applying azadirachtin to different food sources. For example, the dry weight of food eaten by the tobacco hornworm, *Heliothis virescens* , was reduced to 64% of the control value when larvae were fed artificial diet containing 10ppm of azadirachtin (Barnby and Klocke, 1987). Dry weight gain was reduced to 20% of control at this concentration of azadirachtin. Changes in feeding behaviour which occur as a result of eating azadirachtin-treated food have been studied in a few cases. Simmonds and Blaney (1984) showed that larvae of *Spodoptera littoralis* took smaller meals less frequently when offered wheat coated with a 10^{-4}M solution of azadirachtin. Weight gain of caterpillars fed a diet of the same azadirachtin concentration was reduced to 17% of control.

In *Manduca* caterpillars, food intake was not lowered and so the effect on growth was due to a physiological property of the allelochemical. Significant reductions in the efficiencies at which the ingested food was converted to larval biomass (ECI and ECD) were recorded. The overall absorption efficiency of nutrients (AD (dry matter)) was not decreased but the efficiency of nitrogen absorption (AD(nitrogen)) was adversely affected. Arnason *et al.* (1985) showed that AD (dry matter) was unaffected when corn borer larvae, *Ostrinia nubilalis* , were fed artificial diet containing azadirachtin. However, some interference with metabolism after digestion was indicated by a reduced ECD (efficiency of conversion of digested food) value. In a similar study, *H. virescens* larvae have also been shown to exhibit low ECI (efficiency of conversion of ingested food) and ECD values in response to azadirachtin (Barnby and Klocke, 1987). Interestingly, AD (dry matter) for *H. virescens* larvae tended to increase with the concentration of azadirachtin in the artificial diet. Such an increase in AD (dry matter) might have been the result of a decrease in the rate of passage of the food through the gut. It has been suggested from *in vivo* and *in vitro* experiments on locusts that azadirachtin can reduce gut motility (Mordue *et al.*, 1985). No evidence for such an effect

was found for treated *Manduca* caterpillars since the retention times and AD (dry matter) values were the same as controls (Table 7.1).

The value of comparisons between the results of this study and those using azadirachtin-treated foods is questionable. In studies using treated foods, it is not possible to differentiate the effects of azadirachtin on growth after ingestion and those ensuing from reduced feeding. It is certain that reduced food intake would alter the physiological state of *Manduca* and Siegert and Ziegler (1982) showed that starvation lowered the respiration rate of *Manduca* caterpillars. Hence, there appear to be two possible effects which contribute to the growth disrupting ability of azadirachtin. These are costs associated with the metabolism of the insect and reduced nitrogen availability.

Metabolic costs and nitrogen absorption

Azadirachtin caused significant decreases in ECI and ECD. It also produced a slight increase in the respiration rate. Such changes are characteristic of increased metabolic or other costs (see chapter 3). There is growing evidence that plant allelochemicals can induce detoxification activity in insects (reviewed by Dowd *et al.*, 1983). The diversion of energy away from the production of larval biomass and into processes involved with the detoxification could influence the values of ECI and ECD in treated insects. A system of oxidative enzymes, mixed function oxidases (MFOs), has been implicated in protecting phytophagous insects from defensive plant allelochemicals (Gould, 1984). MFOs are associated with the fat body and midgut of insects and the basic detoxification strategy consists of converting lipophilic toxins into polar compounds that can be excreted.

There is evidence that allelochemicals in the food of phytophagous insects can induce higher MFO levels (Yu, 1982). MFO activity has been detected in the midgut and fat body of *Manduca* (Tate *et al.*, 1982) but it is unclear whether this process causes significant demands on the energy budget of insects. For example, MFO activity in velvetbean caterpillars, *Anticarsia gemmatalis*, was increased by 2.8 times when fed cowpeas as opposed to a normal diet of soybeans (Christian and Yu, 1986). This increase did not, however, significantly affect the mean weight of the caterpillars. But in this example, caterpillars may have maintained normal growth by eating more food

and converting it less efficiently into biomass. Schoonhoven and Meerman (1978) found that growth and ECD were decreased in *Manduca* caterpillars fed artificial diet containing 0.1% atropine. From this result, it was concluded that larvae spent a considerable amount of energy detoxifying plant allelochemicals but no measurements of MFO activity were made. Cottee (1984) measured MFO activity in *Locusta migratoria* and *Schistocerca gregaria* nymphs after treatment with azadirachtin and found no indication of increased activity.

Another possible cost might be associated with alterations in the synthesis and secretion of digestive enzymes. Allelochemicals have been extracted from several plant species which can inhibit trypsin-like enzymes (Ryan, 1983). However, Broadway and Duffey (1986a) have recently demonstrated that proteinase inhibitors extracted from soybean and potato increased the production of trypsin in larvae of *Heliothis zea* and *Spodoptera exigua.* Although growth was reduced on diets containing the inhibitors, protein digestion was unaffected. The decrease in larval growth was thought to be related to the cost of elevated trypsin production. In the present work, the activity of a trypsin-like midgut enzyme was found to be decreased to 0.2 times control in azadirachtin-treated *Manduca* caterpillars. Since azadirachtin does not inhibit the enzyme directly, the reduction in activity must have resulted from reduced secretion. However, it remains possible that the synthesis of other enzymes was increased and that extra costs ensued. Unfortunately, no other enzymes were measured in this study.

Treated caterpillars experienced a significant reduction in the amount of nitrogen absorbed from the food. The injection of 25μg of azadirachtin per larva reduced AD (nitrogen) for day 1 caterpillars to 0.7 times control. The growth rate of these insects was reduced to roughly half that of control insects. The limiting effect of protein deprivation was discussed for *Manduca* caterpillars in chapter 4. A similar reduction in growth was observed in the previous study when caterpillars were fed a diet containing 77% of the protein content of normal diet. In other words, a decrease in protein digestion experienced by treated insects has produced a similar effect to reducing food quality. Hence, it seems likely that the disruption of growth in azadirachtin-treated insects could be largely due to a decrease in nitrogen availability.

Changes in the structure and digestive physiology of the gut

Characteristic alterations occurred in the structure of the midgut epithelial cells as a result of treatment with azadirachtin. The apical area of the columnar cells was noticably swollen and there seemed to be a proliferation of secretory vesicles. The effect of azadirachtin on midgut structure has been studied in locusts. Cottee (1984) showed that the midgut epithelial cells of *L. migratoria* nymphs were enlarged after nymphs were injected with 10µg of azadirachtin. Unlike the structural changes in *Manduca*, the enlargement in locusts was not concentrated in the apical area of the cells but the whole cell became swollen. Alterations in the details of the cytoplasmic material of the columnar cells were not examined. In general, the effect of allelochemicals on gut histology has received limited attention. Steinly and Berenbaum (1985) showed that swallowtail caterpillars, *Papilio polyxenes*, exhibited lesions in the midgut epithelium when fed leaves coated with tannins. In *Manduca*, reports of alterations in midgut histology have concerned the endotoxin, *Bacillus thuringiensis*, which causes both goblet and columnar cells to swell (Spies and Spence, 1985).

A number of possible explanations for the decrease in trypsin activity of azadirachtin-treated caterpillars can be suggested. First, enzymes which have been synthesised (probably by the Golgi complex, Santos *et al.*, 1984) might be inhibited from being released into the midgut. In this case, a build-up of enzyme would be expected to occur in the midgut tissue. Such a scheme receives some support from the increased numbers of secretory vesicles observed in the apical blebs of treated insects (Figs.7.6b,7.7b,7.8b). However, only an insignificant increase in trypsin-like activity was found in the midgut wall of treated insects which argues against such a mechanism. Second, trypsin might have been synthesised as an inactive precursor (trypsinogen) and azadirachtin may have interfered with the conversion of the precursor to active trypsin. However, no evidence of trypsinogen activity was found in the midgut. Third, the synthesis of the enzyme itself may have been inhibited. Since the trypsin activity of the midgut wall of treated caterpillars was not different to controls, it seems unlikely that enzyme sy nthesis in the columnar cells was impaired.

There are several possible mechanisms which might control digestive enzyme activity in insects. A neural signal could be sent from centres in the CNS to cells in the midgut where the secretion of enzymes would be stimulated. Neurones are associated with the midgut in *Manduca* (see chapter 6) and messages could be passed to and from the gut *via* the recurrent nerve. Recently, it has been suggested that enzyme secretion in Lepidoptera is influenced by the levels of specific nutrients in the food. These stimulate the secretory cells of the gut to synthesise and release digestive enzymes. For example, Broadway and Duffey (1986b) showed that the level of tryptic activity in larvae of *H. zea* and *S. exigua* was proportional to the protein content of the diet. A similar mechanism controlling invertase and amylase activities in *Catopsila crocale* larvae has been proposed (Christopher and Mathavan, 1985).

Hormonal regulation of digestive enzyme activity might also occur. Indirect evidence for this hypothesis comes from the induction of protease activity and the release of neurosecretory material from the corpora cardiaca in response to feeding in the acridid, *Melanoplus sanguinipes* (Dogra and Gillott, 1971). Many insects, including *Manduca* caterpillars, possess endocrine cells associated with the midgut which might be involved in this kind of system (see chapter 6). Hormonal signals sent from the gut or corpora cardiaca could act directly on the enzyme-secreting cells of the midgut, Alternatively, it is possible that hormones might act indirectly by increasing food intake, with enzyme activity subsequently being increased by a secretagogue mechanism. These ideas are necessarily tentative but it is known that azadirachtin can interfere with the secretory function of the endocrine system of insects. Schlüter and Schulz (1984) found that azadirachtin caused a breakdown in the integrity of cells in the corpora allata and an accumulation of neurosecretory material in the corpora cardiaca of adult Mexican bean beetles, *Epilachna varivestis*. The effects of azadirachtin on moulting have been suggested to be the result of the inhibition of secretion of the moult-inducing prothoracicotropic hormone (Rembold *et al.*, 1982).

Amino acid absorption and the Sibly model of food intake

Azadirachtin did not interfere with the absorption of labelled-glycine from the ingested food in the gut of *Manduca* caterpillars. In both control and

treated insects, 40-50% of the label was removed from the gut within the first hour following the labelled meal. This result is in good agreement with the *in vitro* experiments of Thomas and Nation (1984) who demonstrated that 40-50% of the [^{14}C]-glycine injected into the midgut of several crickets was absorbed within 1h. The rapid uptake of labelled glycine from the gut into the haemolymph also agrees with the prompt increase detected in the level of free amino acids in *Manduca* haemolymph at the end of a meal (see chapter 5).

The absorption of nutrients in caterpillars has been connected mainly with the columnar cells (Cioffi, 1984). *In vitro* studies have suggested that active transport may be involved in the uptake of amino acids in larvae of *Bombyx mori* (Saachi *et al.*, 1981). From the electron micrographs of azadirachtin-treated larvae, mitochondria appeared to be less abundant in the apical blebs of treated insects. Despite this difference, the rate of uptake of [^{14}C]-glycine from the gut was unaffected in treated larvae. This observation suggests either that active transport does not play an important role in the absorption of amino acids or that the observed reduction in mitochondrial abundance was not sufficient to adversely affect the active transport process. Hence, the impairment of nitrogen availability in treated insects is probably not the consequence of reduced absorption of the products of digestion.

The above measurements of amino acid uptake can be used to test a current model of feeding control in animals. Briefly, Sibly (1981) proposed a model which predicted that an animal with continuous access to food ought to feed at such a rate that the retention time of food in the gut was optimised to give the maximum rate of nutrient absorption. Sibly's model is summarised in Fig.7.10 from which it can be seen that if food is retained in the gut for too long or too short a time, the overall nutrient gain will be reduced. In other words an optimum retention time must exist. It should be stressed that although the exact value of this optimal retention time will depend on the shape of the curve of assimilation *vs* time, the conclusion that an optimal value must exist is valid for any likely curve shape (*i.e.* one with a sigmoid shape).

There is some circumstantial evidence that Sibly's model might be applicable to *Manduca* caterpillars. Reynolds *et al.* (1985) showed that the retention time of food in the gut of *Manduca* larvae increased by 1.65 times

during the fifth stadium. Despite this increase, the ratio of the retention time to the relative rate of nutrient absorption remained constant. It can also be predicted from the model that as the retention time decreases, the optimum approximate digestibility (AD) should decrease. In agreement with this prediction, the retention times and AD (dry matter) values decreased when *Manduca* larvae were fed diets in which the nutrient content was diluted with cellulose (see chapter 3).

A direct test of this theory involves measuring the rate of nutrient uptake from the gut and correlating this with the retention time. In the model of Fig.7.10, the maximum rate of nutrient absorption occurs at the tangent to the slope of the curve (*i.e.* the line of greatest slope). In the real data of Fig.7.9, an arrow marks the approximate position of this point which lies between 0 and 1h. However, the calculated retention times for both control and treated insects were considerably greater than this (8-9h, Table 7.1). Therefore, the experimental data do not fit the model. This may have been due to the use of a free amino acid rather than a labelled protein that would have required proper digestion before being absorbed. It is also possible that the optimal retention time is not determined by the flow of amino acids and other nutrients might also be limiting.

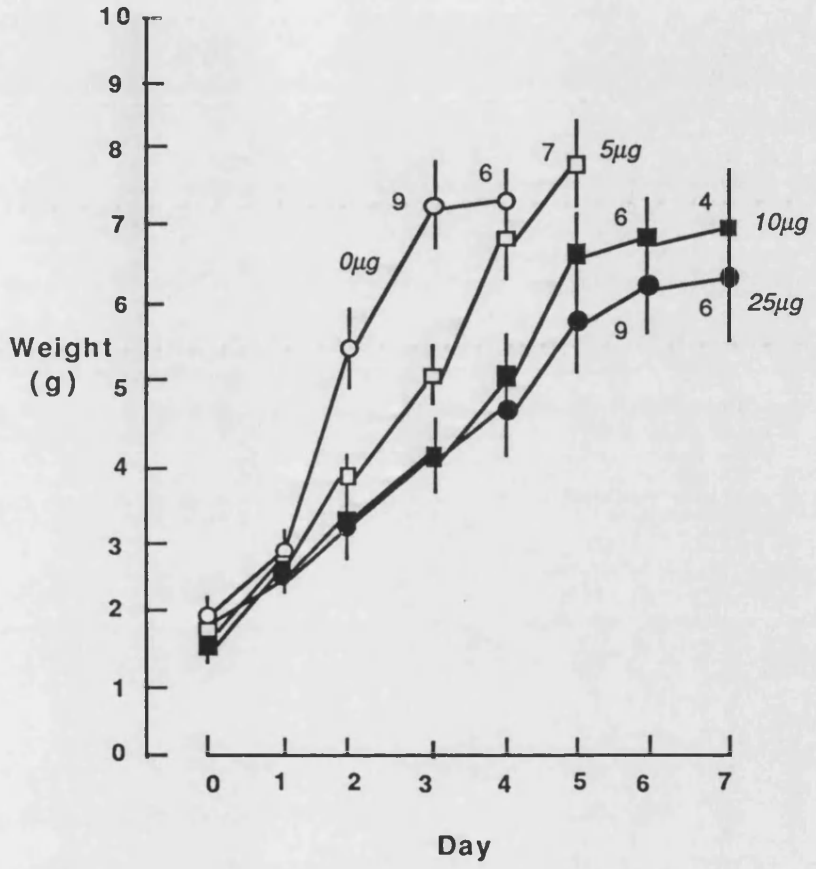
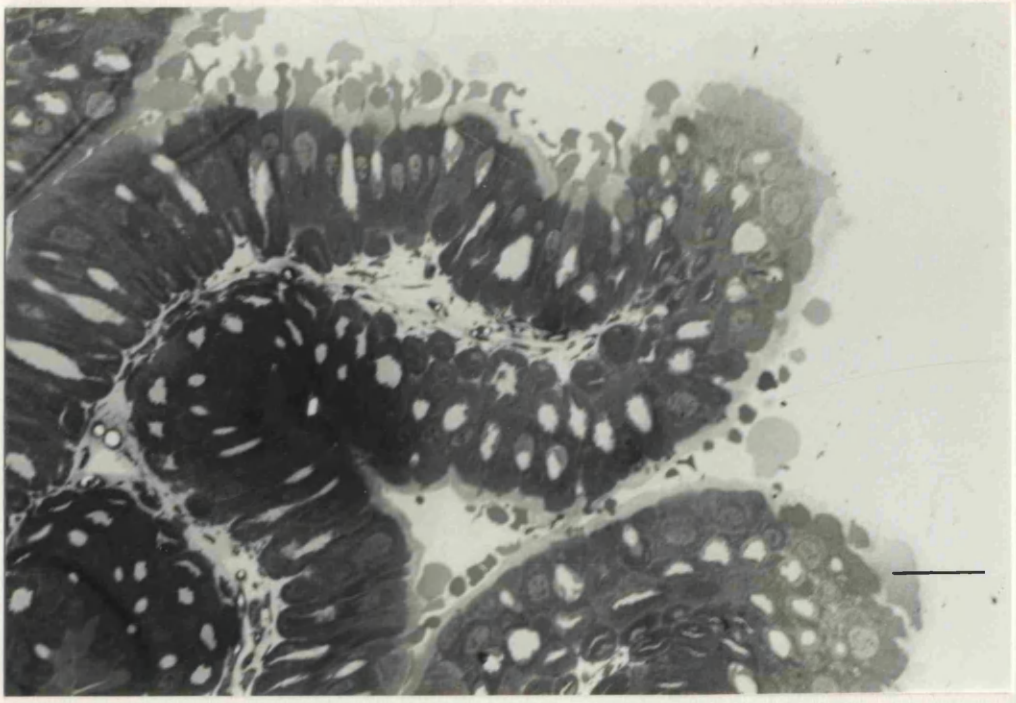


FIG.7.1. Growth of fifth stadium Manduca sexta larvae injected on day 0 with varying doses of azadirachtin (µg per larva). Means ± SE (n=10 per point except where indicated. Smaller sample numbers resulted when some insects ceased to feed and wandered).

FIG.7.2. Sections of midgut epithelium from the anterior region of the midgut of day 1 fifth stadium *Manduca* larvae. (a) control (injected on day 0 with 25µl of 10% ethanol) and (b) azadirachtin-treated (injected on day 0 with 25µl of 1mg/ml azadirachtin in 10% ethanol).
Bar=50 µm (x260).

(a). Control



(b). Azadirachtin-treated

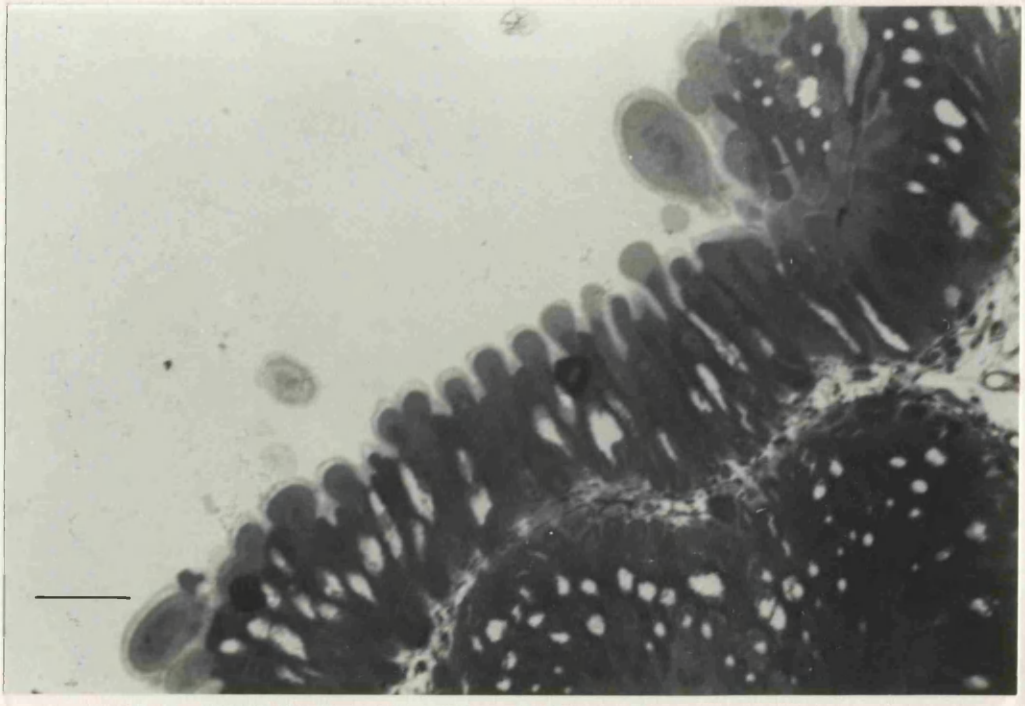
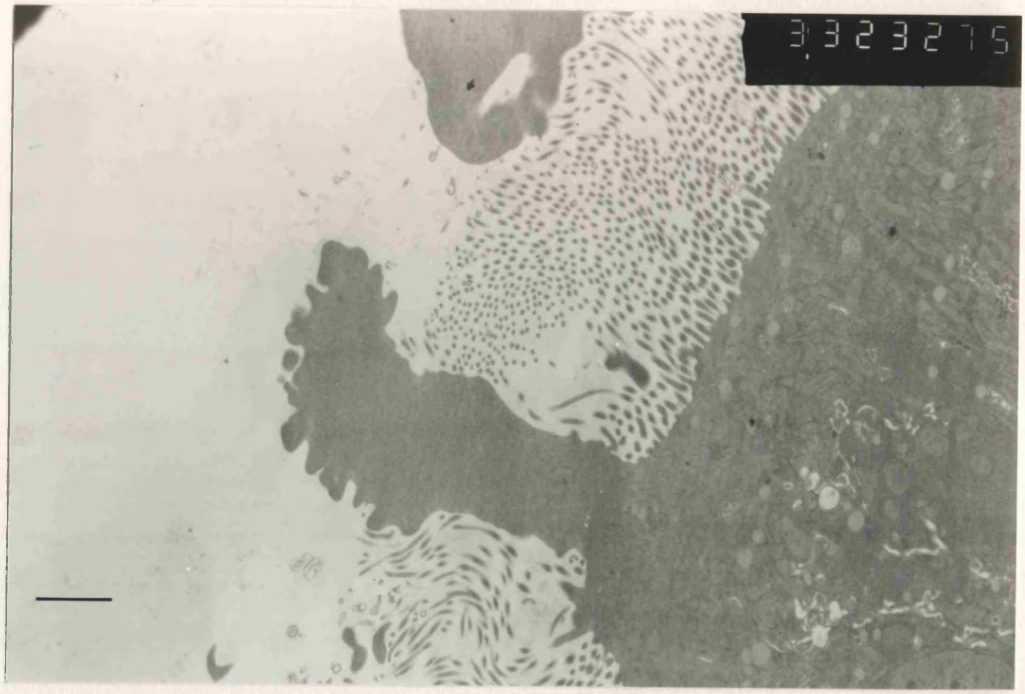


FIG.7.3. Sections of midgut epithelium from the middle region of the midgut of day 1 fifth stadium *Manduca* larvae. (a) control (injected on day 0 with 25µl of 10% ethanol) and (b) azadirachtin-treated (injected on day 0 with 25µl of 1mg/ml azadirachtin in 10% ethanol).
Bar=50 µm (x260).

(a). Control



(b). Azadirachtin-treated

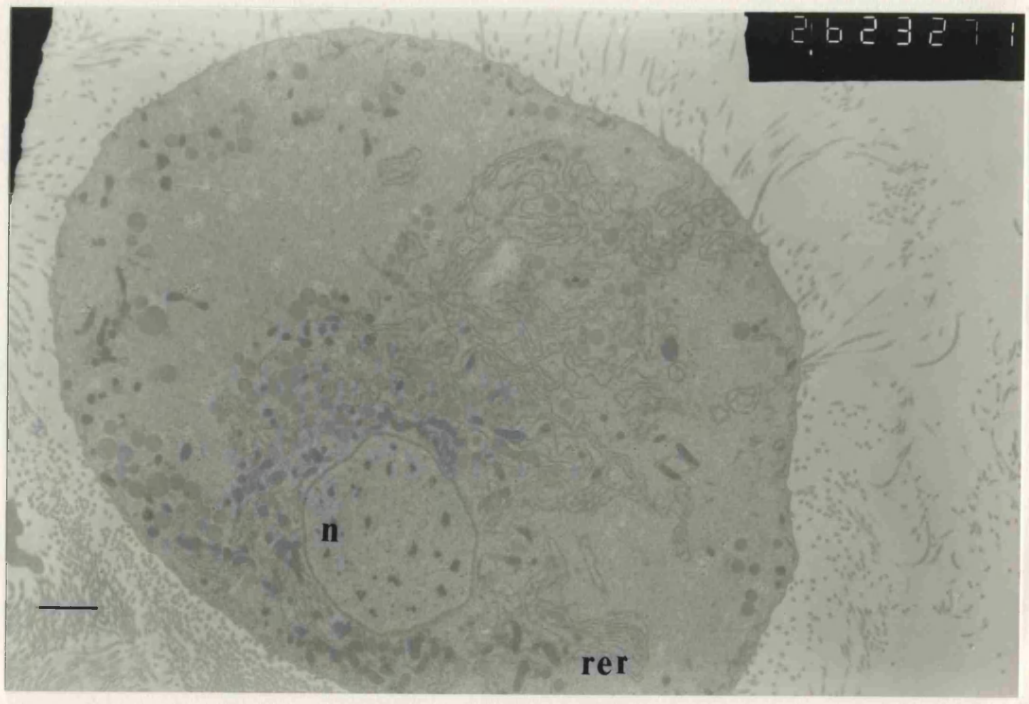
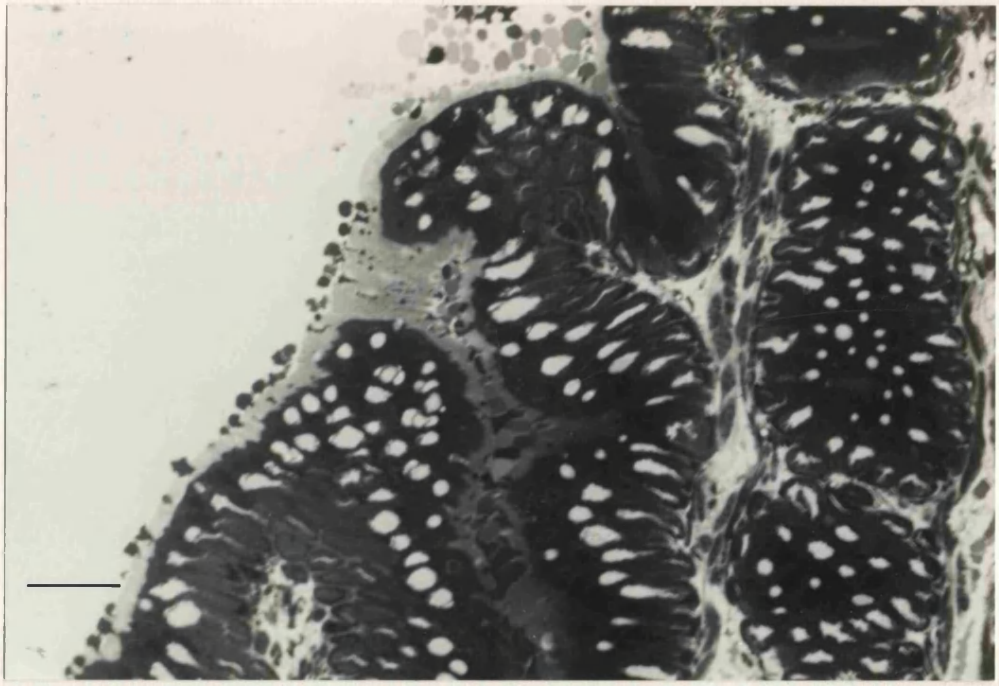


FIG.7.5. Details of apical blebs from columnar cells situated in the middle region of the midgut of day 1 fifth stadium *Manduca* larvae. (a) control (injected on day 0 with 25µl of 10% ethanol; bar=2µm, x5000) and (b) azadirachtin-treated (injected on day 0 with 25µl of 1mg/ml azadirachtin in 10% ethanol; bar=2µm, x4000).
n- nucleus, rer- rough endoplasmic reticulum.

(a). Control



(b). Azadirachtin-treated

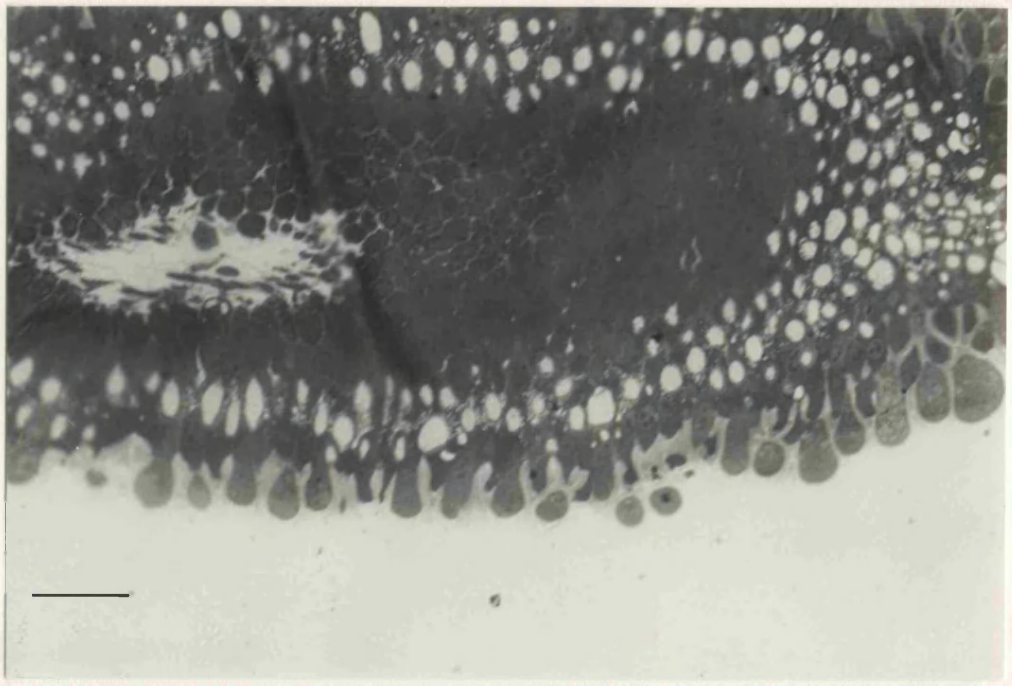
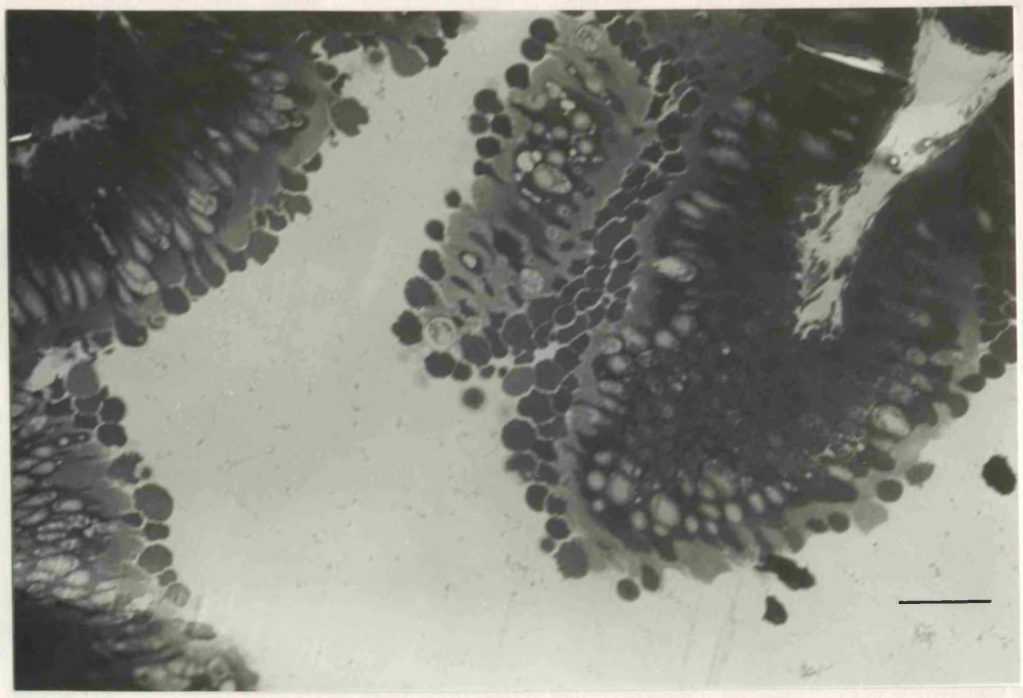


FIG.7.4. Sections of midgut epithelium from the posterior region of the midgut of day 1 fifth stadium *Manduca* larvae. (a) control (injected on day 0 with 25µl of 10% ethanol) and (b) azadirachtin-treated (injected on day 0 with 25µl of 1mg/ml azadirachtin in 10% ethanol).
Bar=50 µm (x260).

(a). Control



(b). Azadirachtin-treated

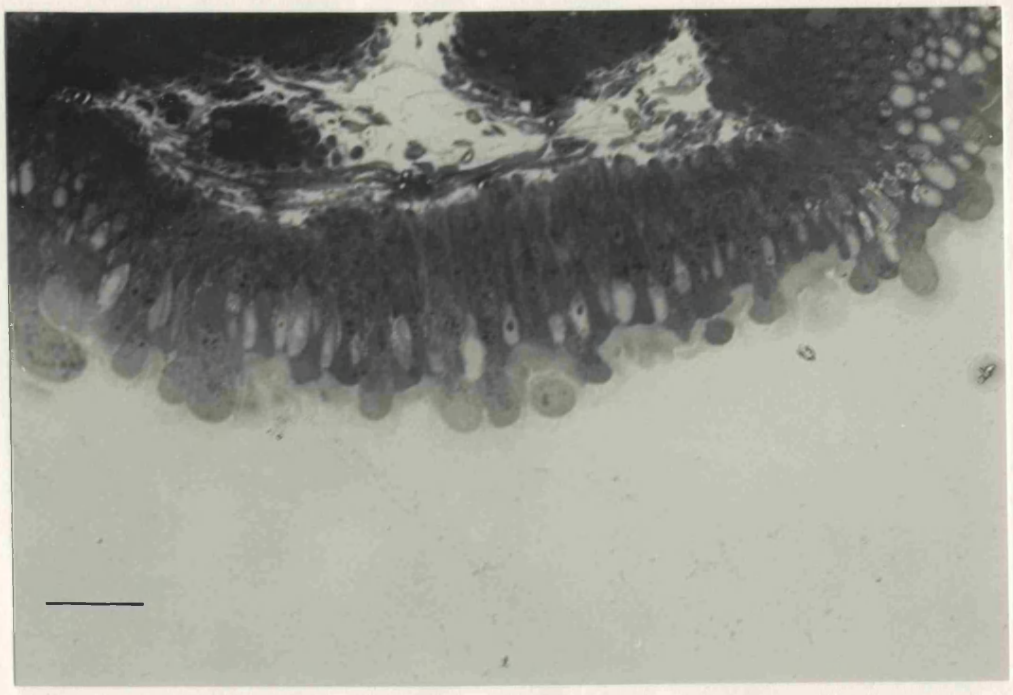
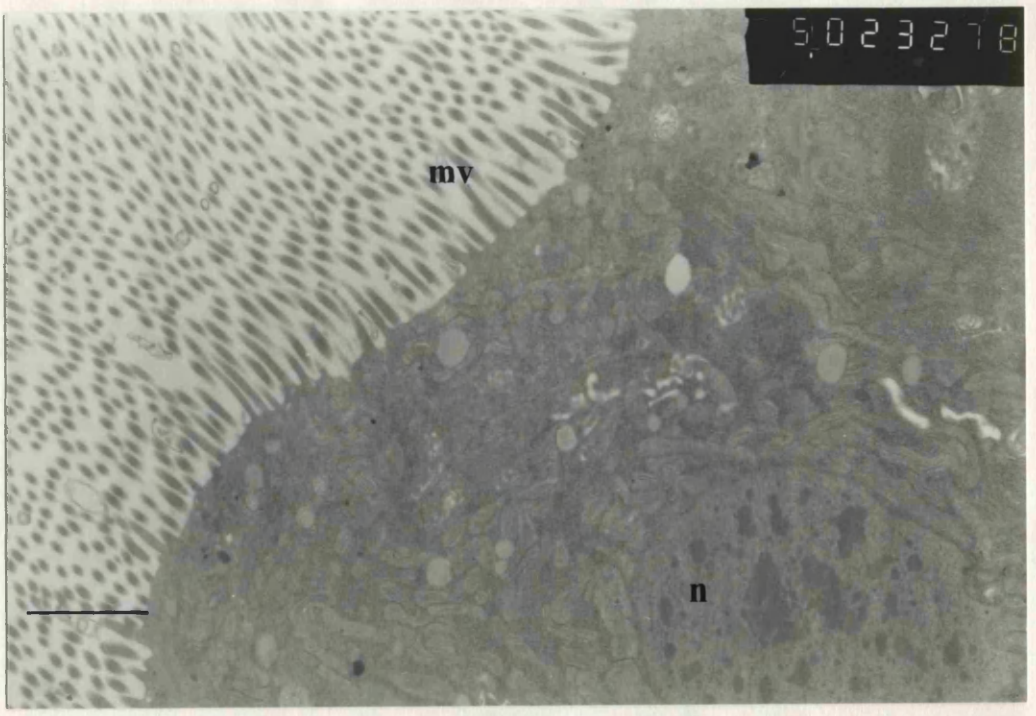


FIG.7.6. Details of columnar cells from the middle region of the midgut of day 1 fifth stadium *Manduca* larvae. (a) apical cytoplasm of control insect (injected on day 0 with 25µl of 10% ethanol; bar=2.0µm, x7500) and (b) apical bleb of azadirachtin-treated insect (injected on day 0 with 25µl of 1mg/ml azadirachtin in 10% ethanol; bar=2.0µm, x4000).
mv- microvilli, n- nucleus.

(a). Control



(b). Azadirachtin-treated

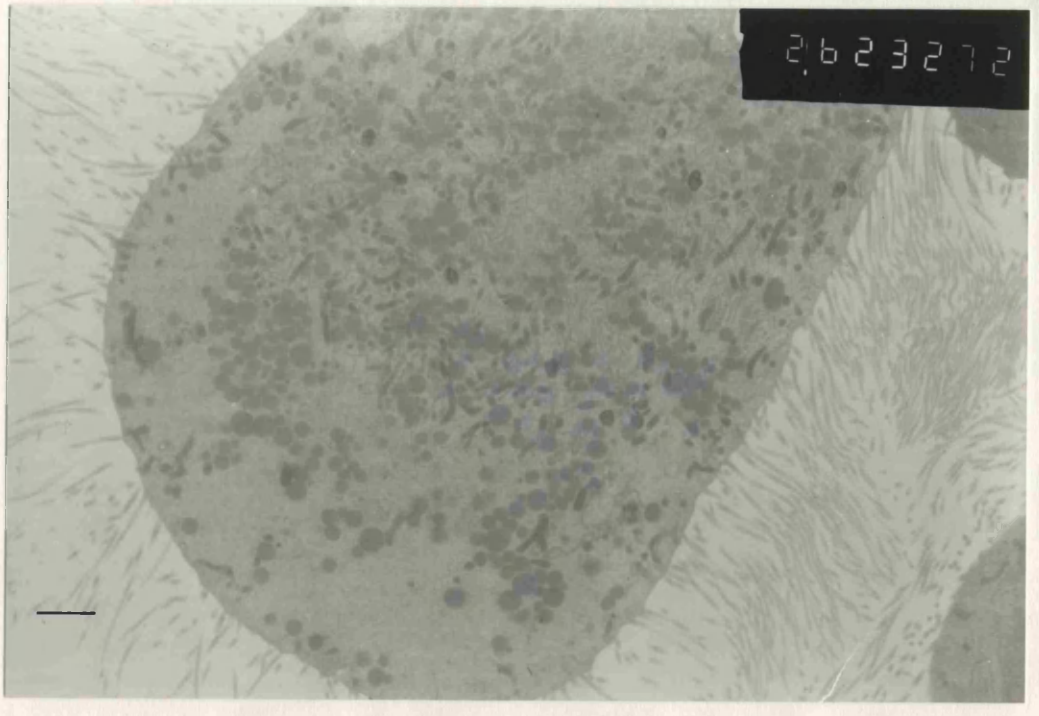
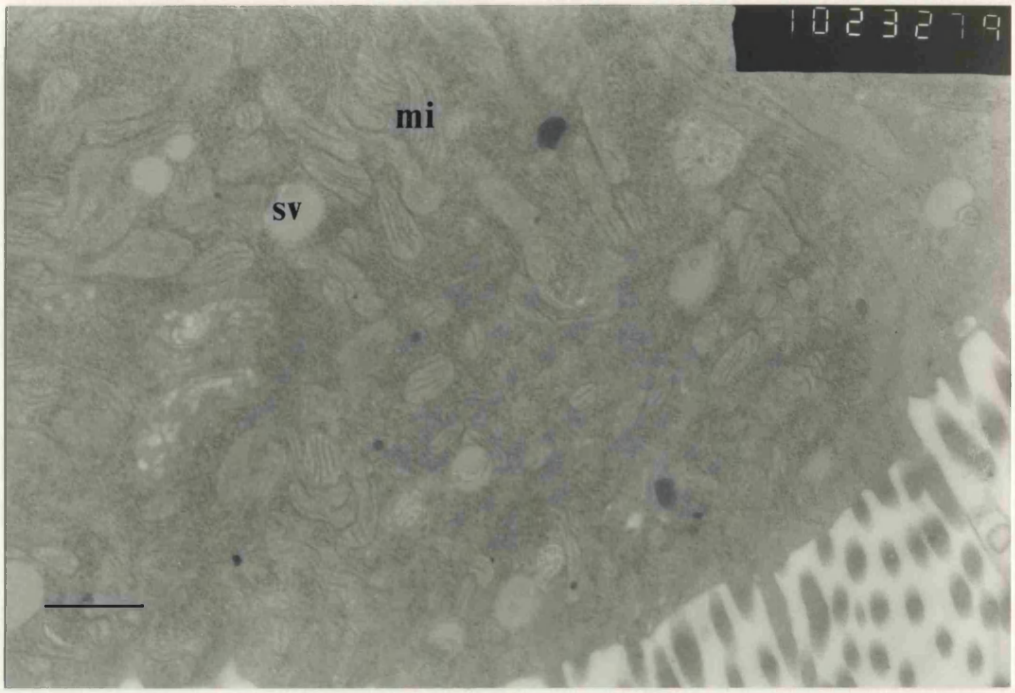


FIG.7.7. Details of columnar cells from the middle region of the midgut of day 1 fifth stadium *Manduca* larvae. (a) apical cytoplasm of control insect (injected on day 0 with 25µl of 10% ethanol; bar=1.0µm, x1200) and (b) apical bleb of azadirachtin-treated insect (injected on day 0 with 25µl of 1mg/ml azadirachtin in 10% ethanol; bar=1.0µm, x7500).
mi- mitochondrion, sv- secretory vesicle.

(a). Control



(b). Azadirachtin-treated

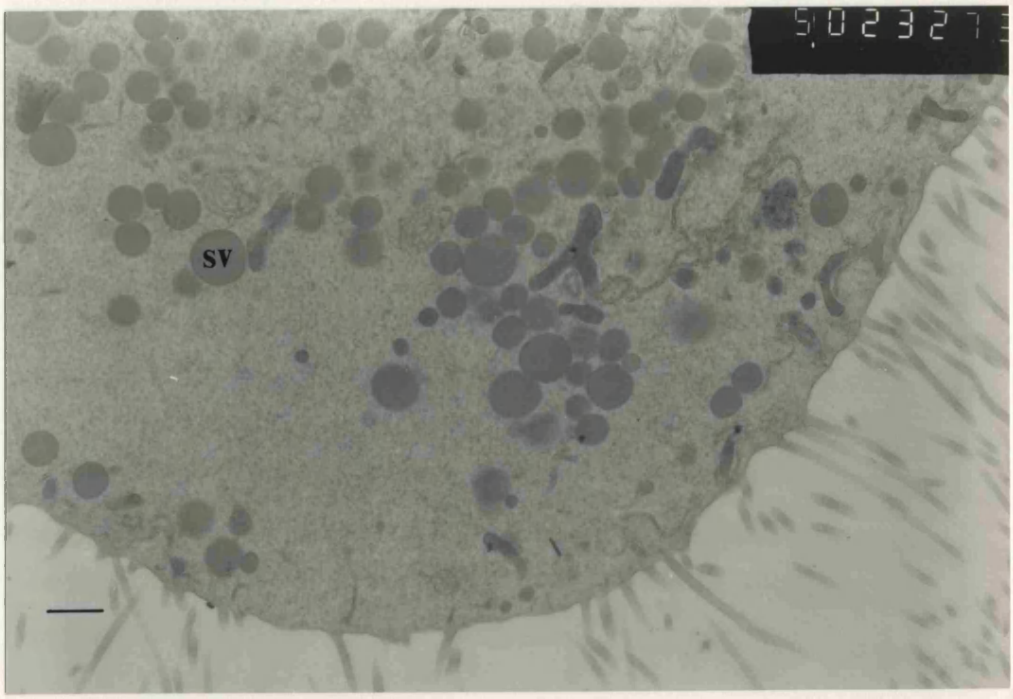
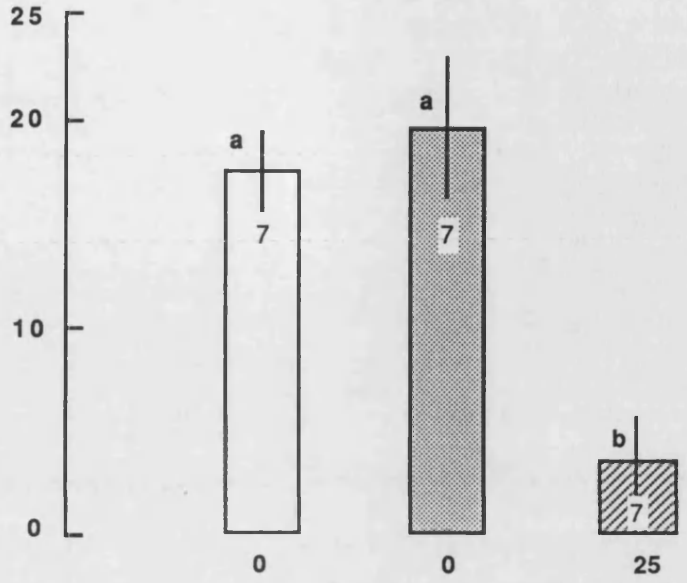
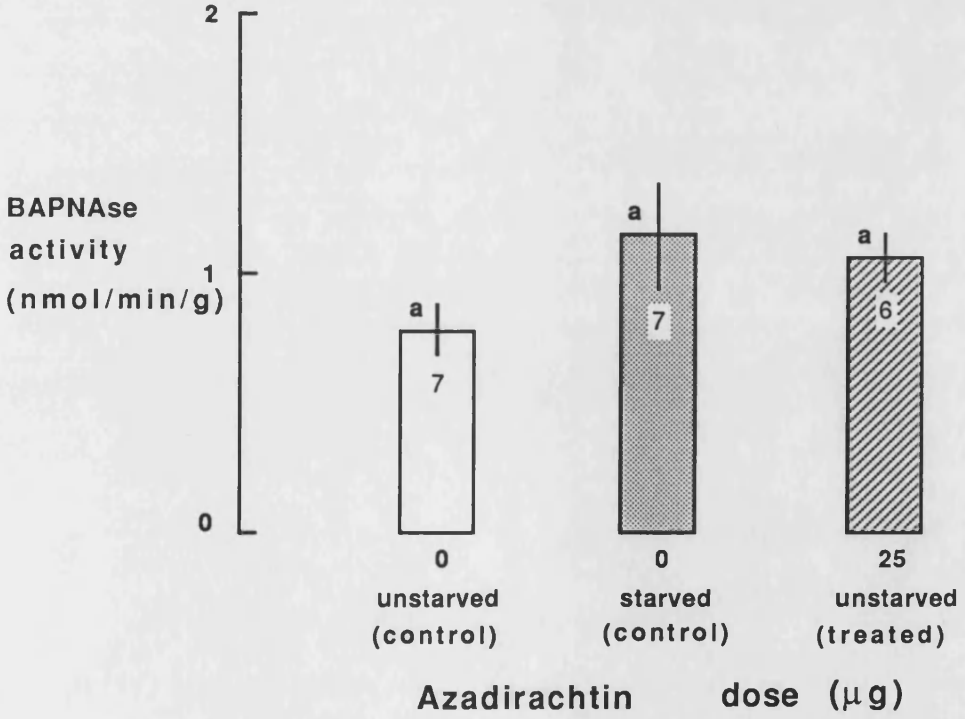


FIG.7.8. Trypsin-like activities in (a) midgut contents and (b) midgut wall of day 1 fifth stadium *Manduca* larvae. Insects were injected on day 0 with either 0µg (controls, 25µl of 10% ethanol) or 25µg of azadirachtin (25µl of 1mg/ml azadirachtin in 10% ethanol). Means ± SE. The number of insects per treatment is given in the open bars. Significant difference between treatments are indicated by different superscripts (one-way ANOVA, 95% confidence intervals based on pooled SD).

(a). Midgut contents



(b). Midgut wall



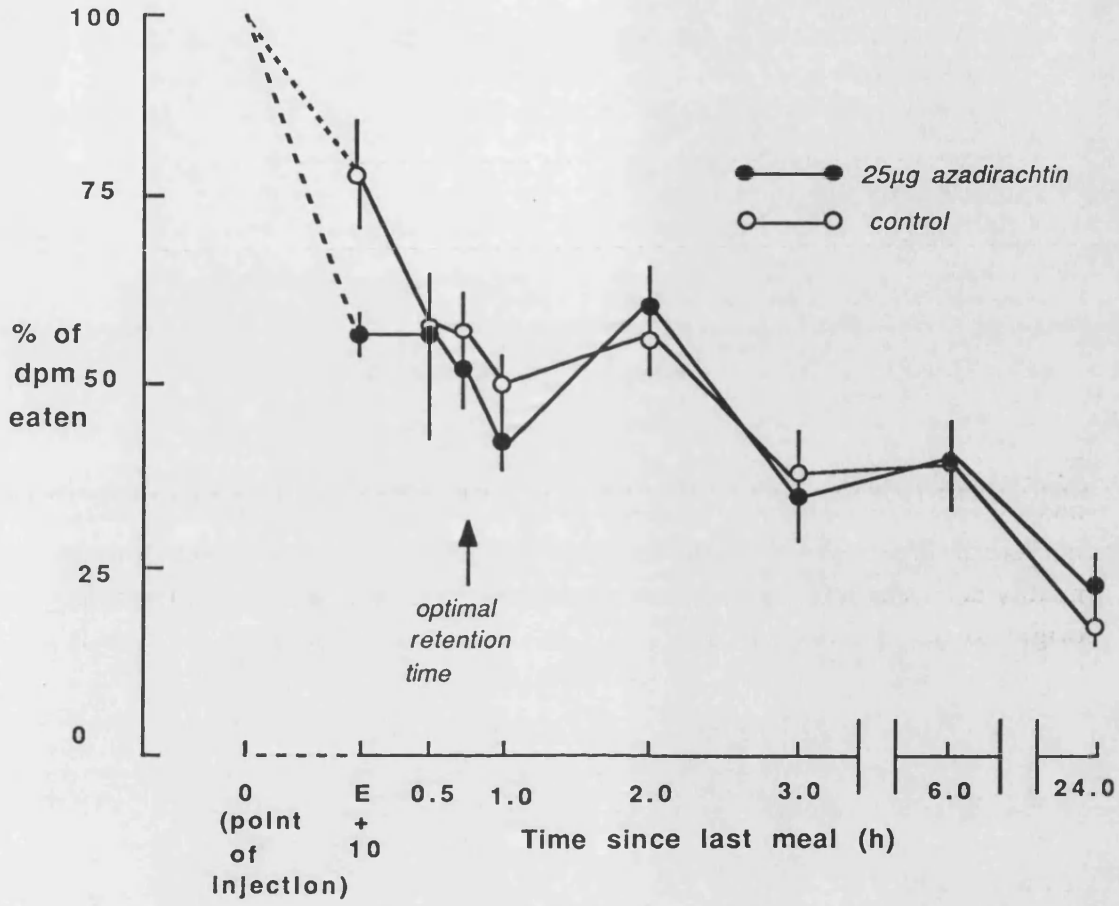


FIG.7.9. The rate of absorption of [14C]-glycine from labelled food in the gut of day 1 fifth stadium Manduca larvae. Insects were injected on day 0 with either 0μg (controls, 25μl of 10% ethanol) or 25μg of azadirachtin (25μg of 1mg/ml azadirachtin in 10% ethanol). The rate of loss of the label is expressed as the % of the original meal present in the gut at intervals after the end of the meal ('E+10', see chapter 5). Means ± SE (n=4-5 per point).

FIG.7.10. A model of the relation between the retention time of a meal in the gut and the uptake of nutrient from it. The rate of uptake is equal to the amount of nutrient absorbed divided by the time that the meal is retained in the gut. This is readily determined for any particular retention time by drawing a line from the origin to that point on the curve. The shape of the curve shown here is hypothetical (although plausible) and is intended only to illustrate the proposition that a unique optimal retention time is likely to exist when the net gain of nutrient from the food will be maximised. This optimal retention time is defined by the point at which the nutrient uptake curve is touched by the line of maximum slope, as shown. The appoximate digestibility (AD) is also optimised at the optimal retention time.

It should be noted that this model only applies to the case where food of equal quality is continuously available to be eaten- a condition which is satisfied for tobacco hornworms feeding on artifical diet. Based on a model by Sibly (1979). After Reynolds *et al.* (1985).

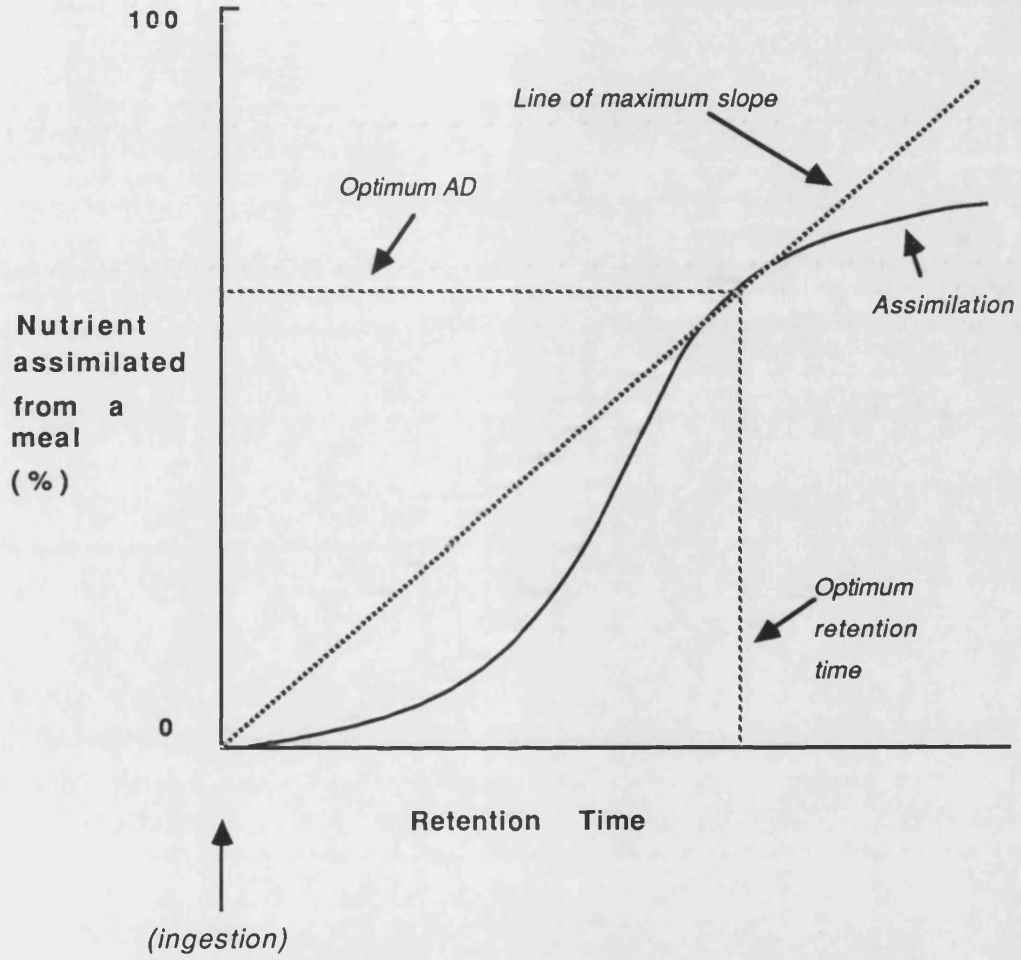

100
Line of maximum slope
Optimum AD
Assimilation
Nutrient assimilated from a meal (%)
Optimum retention time
0
Retention Time
(ingestion)

TABLE 7.1. Nutritional indices for fifth stadium *Manduca* caterpillars (day 0 to day before wandering) and retention times (T_r) for day 1 caterpillars injected with varying doses of azadirachtin. Means ± SE, n=10.

	Azadirachtin dose (µg/larva)			
	0	5	10	25
dry CR (g/day)	0.76 ± 0.05^{a}	0.70 ± 0.04^{a}	0.66 ± 0.02^{a}	0.68 ± 0.05^{a}
dry GR (g/day)	0.25 ± 0.02^{a}	0.25 ± 0.01^{a}	0.16 ± 0.01^{b}	0.12 ± 0.01^{b}
AD	61.06 ± 4.53^{a}	56.38 ± 1.77^{a}	59.34 ± 1.36^{a}	63.92 ± 4.10^{a}
ECI	33.22 ± 2.50^{a}	30.12 ± 1.29^{a}	20.50 ± 1.18^{b}	18.88 ± 2.27^{b}
ECD	46.98 ± 5.64^{ab}	55.29 ± 3.04^{a}	33.98 ± 2.24^{bc}	29.16 ± 4.27^{c}
T_r (h)	7.96 ± 0.50^{a}	4.71 ± 0.39^{b}	6.67 ± 0.82^{a}	8.92 ± 1.39^{a}

Significant differences within rows are indicated by different superscripts (one-way ANOVA, 95% confidence intervals based on pooled SD).

T_r is estimated as dry weight of food in the gut divided by the dry weight of food eaten per h.

TABLE 7.2. Parameters of feeding behaviour in day 1 fifth stadium *Manduca* caterpillars injected on day 0 with 0μg(25μl of 10% ethanol) and 25μg azadirachtin. Means ± SE,n=10.

	Azadirachtin dose (μg/larva)		
	0	25	Significance level of difference
Percentage of time spent feeding (%)	8.34 ± 0.45	7.65 ± 0.59	NS
Bout length (min)	3.95 ± 0.22	4.44 ± 0.25	NS
Gap length (min)	40.20 ± 3.30	51.90 ± 3.80	*
Bout frequency (h^{-1})	1.27 ± 0.08	1.05 ± 0.10	NS
Bout criterion (min)	4.89 ± 1.10	8.10 ± 1.80	NS

* Indicates a significant difference between the means based on a two-sample t-test ($p<0.05$).

Chapter 8

Summary and overview

Summary of findings

A quantitative analysis of feeding behaviour revealed that *Manduca sexta* larvae ate discrete meals when fed tobacco or artificial diet. Evidence for an ability to control food intake was demonstrated by a difference in the time spent feeding on the two types of food. In contrast to locusts (Simpson, 1981; Simpson, 1982), there was no short-term or daily rhythm of feeding activity. The time spent feeding increased throughout the instar and was due to longer bouts and shorter gaps. A similar pattern has been observed in locusts (Simpson, 1982). The dependence of bout length on the preceding gap length and the consistency of the dry weight eaten per meal on either food, suggested that volumetric feedback (Simpson and Bernays, 1983) may be involved in the regulation of meal size.

When larvae were fed artificial diets diluted to varying extents with either cellulose or water, they compensated for the reduced nutrient content by eating more food. Similar responses have been described for several other insects (see chapter 3). Although substantial, the compensation was in most cases insufficient to maintain normal growth rate. Compensation of food intake was achieved by changes in bout length and bout frequency. These results reinforced the earlier conclusion that *Manduca sexta* larvae could control their food intake. In addition, they suggested that both volumetric and nutrient feedbacks may be involved in the control of feeding.

The responses to reduced food quality were further examined using diets in which the carbohydrate, protein and lipid contents were separately diluted with

cellulose. Insects fed a diet containing 77% of the normal protein content initially attempted to increase their food intake (*i.e.* on day 0). This response was characterised by increases in both bout length and bout frequency. In contrast, locusts have been shown to compensate to reduced dietary protein solely by increasing meal frequency (Simpson and Abisgold, 1985). Dilution of the digestible carbohydrate content of the diet did not affect growth or food intake. However, insects fed a diet containing 83% of the normal lipid content, significantly increased their food intake. Overall, the results suggested that nutrient feedbacks might be important in the control of meal initiation and termination.

Haemolymph nutrients have recently been shown to be important in the control of meal initiation in locusts (Abisgold and Simpson, 1987). Changes in blood composition following feeding were monitored in normal-feeding and protein-compensating insects. A dramatic decrease in glucose concentration occurred after feeding with a time course which coincided with the approximate interfeed length of normal feeding insects. It was not possible, however, to demonstrate the involvement of a glucose feedback in the regulation of meal frequency. Interesting differences in haemolymph amino acid concentration were detected in both groups of insects. In locusts, it has been clearly shown that the increase in meal frequency in protein-compensating insects was related to the haemolymph amino acid concentration (Abisgold and Simpson, 1987). However, a similar mechanism controlling compensation in *Manduca sexta* larvae was not found.

The above results indicated that both volumetric and nutrient feedbacks may influence the control of feeding in *Manduca sexta* larvae. These hypotheses were directly tested by artificially manipulating the level of nutrients and/or bulk material in the gut and haemolymph. The results of these experiments indicated that nutrient levels affected the initiation and termination of feeding. There was no evidence for the volumetric control of food intake established in blowflies and locusts (Dethier, 1976; Simpson and Bernays, 1983).

Azadirachtin injected directly into the haemocoel of *Manduca sexta* caterpillars interfered with growth. This effect was related to increased metabolic costs and a decrease in the amount of nitrogen absorbed from the

food. Cellular damage in the midgut and a decrease in trypsin-like enzyme activity were correlated with the reduction in nitrogen availability. Other instances of azadirachtin-induced effects are consistent with an inhibition of secretory activity. For example, Schlüter *et al.* (1985) showed that azadirachtin disrupted the normal pre-moult rise of moulting hormone in *Manduca sexta*. The main target of azadirachtin may, therefore, be the cellular control of secretion.

Synthesis

There are two important conclusions which can be drawn from this study on feeding and growth in *Manduca sexta* larvae. First, caterpillars are able to exert control over their feeding behaviour and regulate both the frequency and duration of meals. Second, a central component of the regulatory processes is the level of nutrients in the insect's body.

Nutrient availability plays an important part in the 'apparency' theory of insect-plant relationships (Feeny, 1975; Rhoades and Cates, 1976). The theory predicts that low nutritional status is a good adaptive strategy for plants. However, the ability of *Manduca sexta* and other insects to compensate for food of reduced nutrient content simply by eating more of it argues against this idea (Moran and Hamilton, 1980). The distinction between nutritional plant defences ('quantitative') and toxins ('qualitative') is in any case becoming increasingly unclear (Fox, 1981). Indeed, azadirachtin (a typical 'qualitative' defence) has been shown here to interfere with nitrogen availability and thus growth. Hence, the availability of nutrients in the insect's food is important in terms of a general theory of insect-plant interactions and in the physiological regulation of feeding in *Manduca sexta* larvae and other insects (Abisgold and Simpson, 1987). Unfortunately, time constraints precluded a more detailed investigation of the physiological mechanisms underlying the effect of nutrient injections on feeding behaviour in *Manduca* larvae.

Many interesting questions remain unanswered. Are volumetric feedbacks from the hindgut important? Are hormones involved in the control of feeding? Which specific nutrients affect meal size and frequency and how is this effect mediated? What is the central mechanism controlling feeding? Does azadirachtin interfere with the general control of secretory activity? Until the

present study, the behavioural and physiological organisation of feeding in caterpillars has not been closely examined. The present work represents an initial attempt to fill this gap. Clearly, there is considerable potential for further productive studies.

Appendix 1

1. Maintenance of the Manduca culture

Larvae were reared in a controlled environment room at 25 ± 0.5 ^{0}C, 50 ± 10% relative humidity and a diapause-averting (LD 17:7h) photoperiodic regime. The lights were switched on at 7 a.m. and off at 12 p.m. The larvae were fed from hatching on artificial diet which was slightly modified from that described by Bell and Joachim (1976).

Eggs were collected daily from the adult box and allowed to hatch in large (*c.* 200ml) plastic pots. Hatching took about 5 days and newly hatched first instar larvae were placed on cubes of diet (*c.* 7.0g) in small plastic pots (*c.* 30ml) with tight-fitting lids. Animals remained in these pots until ecdysis to the fifth instar. Newly moulted 'fifths' were placed in large (*c.* 200ml) plastic pots with more fresh diet (*c.* 25g). Insects ceased to feed after about 5 days of the fifth instar and began to search for a pupation site ('wandering'). Wandered animals were placed in holes (*c.* 10cm by 2.5cm diameter) drilled in wooden blocks. The holes were fitted with corks and pupal development proceeded with pupae ecdysing about 10 days after wandering. They were then placed in plastic trays until they developed into pharate adults (distinguishable by their dark appearance and thinning of the cuticle). Pharate adults were placed in a tray in the adult box with a wire mesh frame provided so that newly eclosed adults could hang freely to expand their wings. The adult box (*c.* 1m^3) was maintained under long day conditions and constant illumination was provided by a dim light bulb (4W). A leaf mimic was used as an oviposition mimic (disposable nappy liner impregnated with tobacco extract). Adults fed from a container of sucrose solution (10%) attached to a

hollow, yellow, plastic flower.

2. Artificial diet

(i) Ingredients

Premix (normal type)	g
casein (BDH)	420
wheatgerm (grocery)	900
sucrose (grocery)	360
dried bakers yeast (grocery)	180
Wesson's salts (Uniscience, ICN)	120
choline chloride (Sigma)	12
sorbic acid (Sigma)	18
cholersterol (Sigma)	24

Other ingredients (normal)	
Agar	40g
10% formaldehyde	8.0ml
ascorbic acid	8.0g
aureomycin (Lederle)	0.2g
linseed oil (Polycell)	4.0ml
corn oil (grocery)	4.0ml
Vanderzant vitamin mixture (Uniscience, ICN)	0.2g

(ii) Preparation

336g of premix was mixed with 700ml of boiling water in a foodmixer (Kenwood). 25g of agar was mixed with 1000ml of distilled water, cooked in a microwave oven for 15min, and added to the premix in the blender. While this mixture was being mixed, linseed oil (4.0ml), corn oil (4.0ml) and 10% formaldehyde (8.0ml) were added. The diet was allowed to cool to below 7∞C and 0.2g of Vanderzant vitamin mixture, and 0.2g aureomycin added. The mixture was mixed thoroughly. The diet was poured into a foil-lined pan,

and allowed to set.

3. Ephrussi and Beadle (1936) insect saline

	g/litre
NaCl	75
KCl	3.5
CaCl2.2H2O	2.1

composition of final saline

	mM
Na+	128
K+	5
Ca2+	2
Cl-	135

A 10x stock solution was prepared which was diluted for use as necessary.

Appendix 2

1. Data collection program

Semantics:

The time (in seconds) since the start of the program is outputted to disk whenever the numbered keys (0-9) are pressed. A flashing symbol on the display indicates which insects are feeding at any time.

Syntax:

```
  10 CLS
  20 DIM INSECT (10)
  30 PRINT TAB (3,10); "FEEDING BEHAVIOUR PROGRAM
(ATOO1)"
  40      PRINT      TAB      (3,11);
"                                    "
  50 PRINT TAB (3,14);"    BY ANDREW TIMMINS AP&E"
  60 PRINT TAB (3,25);"    PRESS 'C' TO CONTINUE"
  70 REPEAT
  80 UNTIL GET=67
  90 CLS
  100 INPUT "FILENAME",Z$
  110 PRINT TAB (12,7); "INSECT NUMBER"
  120 PRINT TAB (2,10); "1"; TAB (6,10); "2"; TAB
(10,10);
  "3"; TAB (14,10); "4"; TAB (18,10); "5"; TAB
(22,10);
  "6"; TAB (26,10); "7"; TAB (30,10); "8"; TAB
(34,10);
```

```
  "6"; TAB (26,10); "7"; TAB (30,10); "8"; TAB
(34,10);
  "9"; TAB (38,10); "10"
  130 PRINT TAB (1,13); CHR$ (136)
  140 PRINT TAB (0,2); "PRESS 'S' TO STOP"
  150 PRINT TAB (0,3); "PRESS 'P' TO PAUSE"
  160 PRINT TAB (0,4); "PRESS 'C' TO CONTINUE"
  170 VDU 23,1,0;0;0;0;
  180 Y=OPENOUT Z$
  190 TIME=0
  200 A$=GET$
  210 IF A$="S" THEN 350
  220 IF A$="P" T=TIME:PRINT TAB (1,30);
  "PAUSE":A$=GET$:
  PRINT TAB (1,30); "ANALYSING";:TIME=T    230 IF A$
   <"0" OR A$ >"9" THEN 200
  240 A=VAL (A$)
  250 IF A=0 THEN A=10
  260 INSECT(A)=1-INSECT(A)
  270 X$="F"
  280 IF INSECT(A)=0 THEN X$=" "
  290 PRINT TAB (4*A-2,13);X$
  300 BEHAV$=A$+" "+SR=TR$ (TIME DIV 100) +CHR$ (13)
   +CHR$(10)
  310 FOR 1%=1 TO LEN (BEHAV$)
  320 BPUT£Y,ASC (MID$(BEHAV$,1%,1))
  330 NEXT 1%
  340 GOTO 200
  350 CLOSE£Y
  360 CLS
  370 END
```

2. Sorting programs (S)

These programs are based on the C programming language and make use of the pattern matching programs (*e.g.* awk, grep) available on the UNIX system (Bourne 1983).

Semantics:

A pattern matching filter sorts the data into separate records for each insect and the output is sent to separate files.

Syntax:

```
cat $1 | grep "^0 " > $1.0
cat $1 | grep "^1 " > $1.1
cat $1 | grep "^2 " > $1.2
cat $1 | grep "^3 " > $1.3
cat $1 | grep "^4 " > $1.4
cat $1 | grep "^5 " > $1.5
cat $1 | grep "^6 " > $1.6
cat $1 | grep "^7 " > $1.7
cat $1 | grep "^8 " > $1.8
cat $1 | grep "^9 " > $1.9
```

(ii) S2

Semantics:

Bouts and gaps are separated from each other and are stored in a new file.

Syntax:

```
cat $1 | awk'
BEGIN {
        N=0
}
{
        print $2-N > "all"
        N = $2
}'
mv all $1.all
```

(iii) S3

Semantics:

Bouts and gaps are removed from the same file and stored separately.

Syntax:

```
cat $1 | awk'
     if (NR % 2 == 0) print $1 > "bout"
     else print $1 > "gap"
}'
mv act $1.bout
mv inact $1.gap
```

(iv) S4

Semantics:

Time values are converted from seconds to minutes.

Syntax:

```
cat | awk '
{
     printf "%2.2f\n", $1/60
}'
```

(v) S5

Semantics:

Gap intervals are incremented at 0.5 min intervals and the number of intervals greater than each increment is calculated together with the log10 of this number (these values are plotted against each other and printed out using MINITAB 5.1 to produce log-survivor functions).

Syntax:

```
cat $1 | awk '
{
      for (i=0.5; i<=60.0; i=i+0.5)
                                                  if  ($1  >
i) res[i] += 1
}
END {
      for (i in res)
                                                  print  i  "
 " res[i] "    "log(res[i])
} ' | sort -n | awk '
BEGIN {
      N = 0
}
{
      if (N == 0 || $2 != N) print $1 "     " S2 "   "
$3
      N = $2
} '
```

(vi) S6

Semantics:

Bouts which are separated by a gap less than the bout criterion are added together.

Syntax:

```
finclude <stdio.h>

main(argc, argv)
int argc;
char *argv[];
```

```
   {
        int i1, i2, i3;
        int num;

        num = atoi(argv[1]);

        scanf("%d\n", &i1);
        printf("%d\n", i1);       /* Read in and re-
print first
                                          gap    */

/* value. */

        scanf("%d\n", &i1);     /* Read first bout */
        while (1)
        {
                                          if
(scanf("%d\n", &i2) == EOF
                                          {

       printf("%d\n", i1);

       exit (0);
                                          }
                                          if
(scanf("%d\n", &i3) == EOF)
                                          {

       printf("%d\n%d\n", i1, i2);

       exit (0);
                                          }
                                          if  (i2  <
num)
                                          {

       i1 = i1 + i3
```

```
                                                    }
                                                    else
                                                    {

        printf("%d\n%d\n", i1, i2);

        i1 = i3
                                                    }
        }
}
```

(vii) S7

Semantics:

Gaps which are less than the bout criterion are removed from the files containing gap lengths.

Syntax:

```
cat $1 | awk '
{
        if (0+$1 > 0+x) print $1
} ' x=$2 -
```

(viii) S8

Semantics:

The % time spent feeding is calculated for each insect.

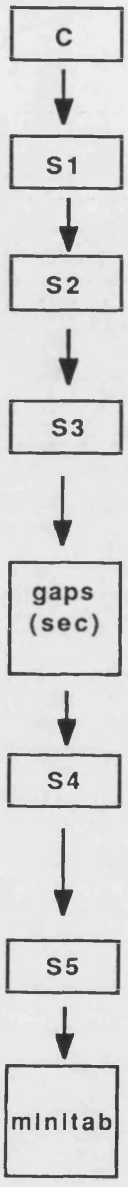
Syntax:

```
cat $1 | awk '
BEGIN {
        N=0
}
```

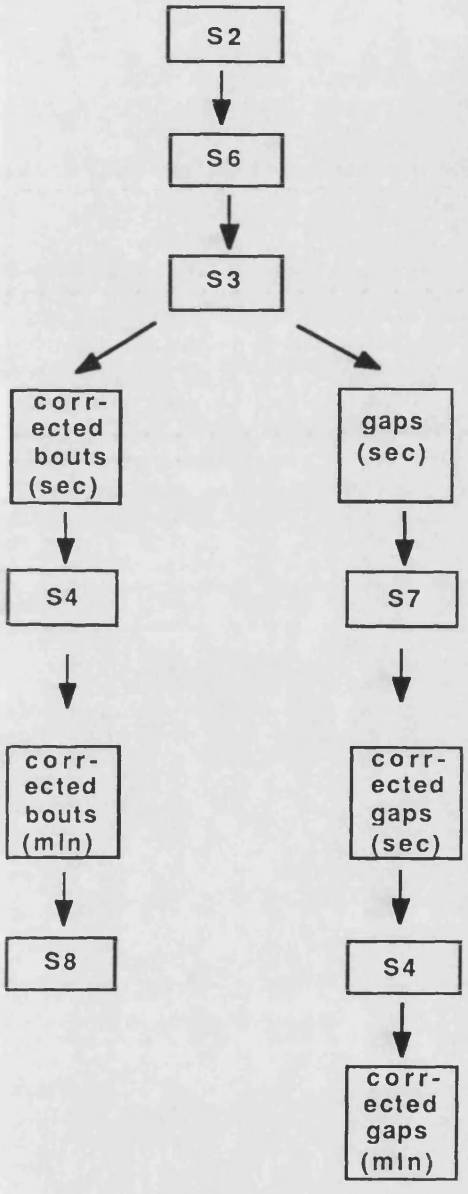
```
{
      N = N + $1
}
END {
      print N *100/720
} '
```

3. Flow charts

(i) to produce log survivor functions



(ii) to correct data for bout criteria



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